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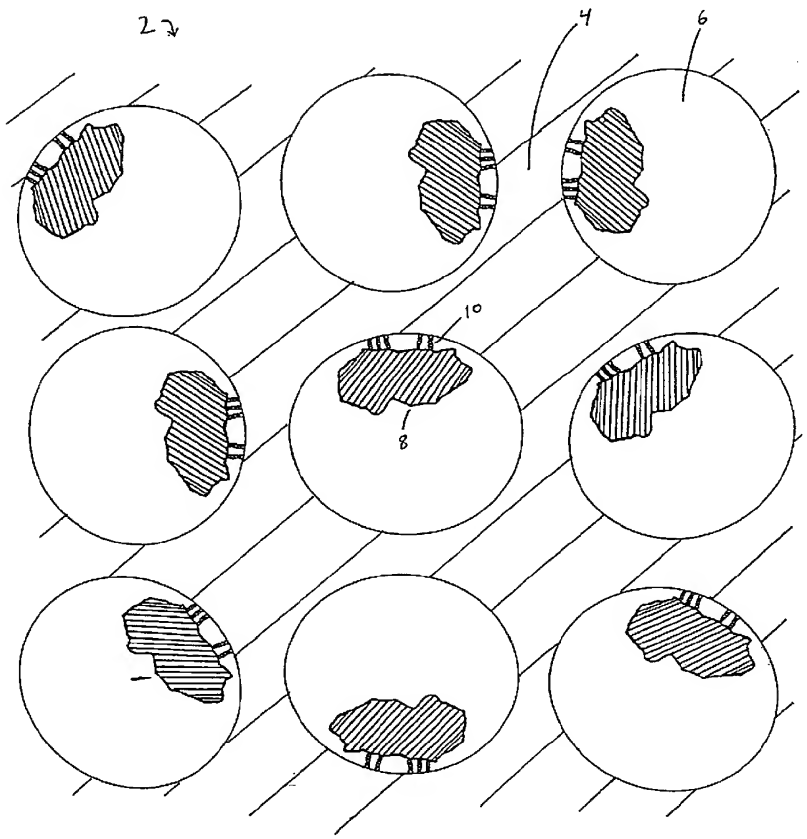
(54) Title: PROTEINS IN A POROUS SUPPORT



(57) Abstract: A protein system is described in which a protein is bound within a matrix material that has pores that are sized to achieve excellent properties such as: activity, protein density, and stability. In a preferred embodiment, the pore sizes range from 50 to 400 A. One protein that has demonstrated surprisingly good results in this system is OPH. This protein is known to degrade organophosphorus compounds such as are found in chemical weapons and pesticides. Novel methods of forming the protein system and methods of making OPH are also described. Also described is a protein system including a porous matrix material and a protein disposed within the porous matrix material; wherein the protein system contains at least 0.01 mmol of protein per gram of matrix material and exhibits an activity at least 2 times greater that the activity of a protein system that has been formed under identical conditions on a normal silica matrix material. Methods of making protein systems with noncovalent bonding to entrap proteins in the porous matrix are also described.

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PROTEINS IN A POROUS SUPPORT

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Ser. No. 09/791,138, filed February 21, 2001, which is incorporated herein as if reproduced in full below.

FIELD OF THE INVENTION

The present invention relates to proteins in porous supports, methods of supporting proteins, and methods of using supported proteins. The invention also provides an improved method for making organophosphorous hydrolase ("OPH").

BACKGROUND OF THE INVENTION

The usefulness of proteins for facilitating chemical reactions outside biological organisms has long been known and used to great advantage. There is the potential for much greater use of proteins in facilitating a much larger variety of reactions and facilitating these reactions on a larger scale. However, there are many challenges to be overcome before this potential can be fully realized. These challenges include: the need for highly active protein systems; the need for protein systems that maintain high activity under a range of conditions; and the ability to densely pack active protein onto a porous support.

One example of a protein that is useful for catalyzing a variety of useful reactions is organophosphorous hydrolase, ("OPH"). OPH is an enzyme that might be used to inactivate chemical weapons or organophosphorous pesticides. Chemical weapons (*i.e.* nerve gases, especially sarin and VX) and organophosphorous pesticides (*e.g.* parathion, paraoxon and acephate) are highly toxic to higher organisms. Therefore, there is a need for methods of cleaning up undesirable discharges of the chemical weapons and organophosphorous pesticides in accidental spills or production plant contamination. The OPH enzyme offers the potential to inactivate chemical weapons or organophosphorous pesticide without the need for complex and expensive incineration facilities. Despite its potential, the lack of suitable methods for the large scale production of systems with active and stable OPH have limited the application of this enzyme.

The present invention provides improved protein systems that can better meet the challenges described above. Although the invention generally applies to immobilized enzyme systems, etc., in some specific examples, the invention also provides an improved method for making OPH and systems containing active OPH.

SUMMARY OF THE INVENTION

One concept of the invention is the engineering of support structures that match protein sizes to support structure pore sizes. It has been surprisingly found that well-matched sizes can produce protein systems having desirable qualities such as high activity, enhanced stability, and a relatively high density of active protein. Coupling of proteins in pores that are either too small or too large results in inferior properties. Other factors, such as surface area, pore density, pore uniformity and distribution, protein population within a support, and type and density of cross-linking sites may also be utilized to control the characteristics of the protein system.

In one aspect, the invention provides a protein system for use in facilitating chemical reactions. The system includes a porous matrix material that has pores within a solid matrix. In another aspect, the protein system comprises: a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, and a chemically-active protein bonded to the matrix material. "Bonded" refers to covalent, ionic and/or electrostatic attachment to the matrix material. In preferred embodiments, the protein is covalently bonded to the matrix through coupling groups.

In another aspect, the protein system comprises: a porous matrix material being sized such that the protein system comprises 0.01 to 1 mmol of protein per gram of matrix material and wherein the protein in the protein system exhibits an activity of at least 65% that of the activity of the protein in the active state.

The invention further provides a protein system comprising a porous matrix material containing an entrapped protein wherein the protein system is characterized by having at least 0.01 mmol of protein per gram of matrix material and an activity that is at least 2 times greater than the activity of a protein system that has been formed under identical conditions except on a normal silica matrix material. "Normal silica" is uncoated silica bulk material with a pore size of 300 Å with a 12 micrometer bead size; if available the normal silica should be purchased from PolyLC Inc., Columbia Maryland, USA, item #BMSI 1203.

In another aspect, the invention provides a protein system comprising a porous matrix material containing an entrapped protein wherein the protein system is characterized by having at least 0.01 mmol of protein per gram of matrix material and enhanced stability as defined by the stability testing procedures described herein.

The invention also provides a method of forming a protein system comprising the steps of:

providing a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, and reacting the porous matrix material with a protein so that the protein chemically bonds to the porous matrix material. Preferably, this method comprises a step of reacting the porous matrix material with a cross-linking agent to form a porous matrix material having cross-linking agents covalently bound to the surface, and reacting the porous matrix material having cross-linking agents covalently bound to the surface with a protein so that the protein chemically bonds to the porous matrix material.

In another aspect, the invention provides methods in which a protein system is made by adding a protein to a porous matrix material without a cross-linking agent. It has been found that stable and active protein systems can be obtained in which the protein is entrapped by non-covalent bonding. In one such method, a protein system is formed by: providing a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, wherein the porous matrix material has a functionalized surface, and adding a protein so that the protein is entrapped by non-covalent bonding in the porous matrix material.

The invention also provides a method of making OPH. In this method, a host cell is transfected with a vector comprising a sequence encoding OPH, the sequence being operably linked to a T7 expression control sequence. The transfected host cell is cultured under conditions permitting expression under the control of the expression control sequence. The OPH is purified from the cell or the medium of the cell.

The protein system is engineered to match the size of the individual protein with the size of the individual pores, in preferred embodiments, the volume of the individual protein occupies between 5 and 40 % of the average volume of each pore.

The invention also includes methods of using these systems in facilitating chemical processes (i.e., processes of making chemicals) such as hydrolysis, oxidation, hydrogenation, and proteolysis. The invention also encompasses the use of active enzymes in porous supports in filtration equipment for individual soldiers, pesticide workers, vehicles, aircrafts, ships and buildings such as civilian and military defense shelters, to perform detoxifications.

Various embodiments of the present invention can provide numerous advantages including: high protein activities on a porous support; stability under a variety of conditions; high densities of active protein; capability in industrial-scale applications; and providing environmentally safe methods of destroying chemical weapons and organophosphorous pesticides, and avoid the dangers inherent in burning these materials. Other advantages can be envisioned in view of the following descriptions and examples.

BRIEF DESCRIPTIONS OF THE DRAWINGS

FIG. 1 is a conceptionalized, cross-sectional representation of an enzyme disposed in a porous substrate.

FIG. 2 is a ribbon diagram for OPH plasmid.

FIG. 3 is the relevant DNA sequence from the construct's BamH I to Bgl II site that encompasses the region immediately preceding the T7 promoter to just beyond the OPH stop codon, SEQ ID: 2.

FIG. 4 is the OPH amino acid sequence, SEQ ID: 1.

FIG. 5 shows a comparison of Normal Silica and NH_2-SAMMS for OPH immobilization.

FIG. 6 shows an ionic strength effect on OPH entrapped in HOOC-SAMMS.

FIG. 7 shows the effect of pH on OPH entrapment in HOOC-SAMMS.

FIG. 8 shows immobilization efficiency of OPH in HOOC-SAMMS at different pH.

FIG. 9 shows an ionic strength effect on OPH immobilization efficiency in HOOC-SAMMS.

FIG. 10 illustrates stability of OPH immobilized in NH_2-SAMMS.

FIG. 11 shows a comparison of Normal Silica and NH_2-SAMMS for Glucose Oxidase (GOD) immobilization.

FIG. 12 illustrates a coverage effect of a functional group of NH_2-SAMMS on GOD entrapment.

FIG. 13 shows the effect of pH on GOD entrapment in NH_2-SAMMS.

FIG. 14 shows immobilization efficiency of GOD in NH_2-SAMMS at different pH.

FIG. 15 illustrates the stability of GOD immobilized in NH_2-SAMMS.

FIG. 16 shows a comparison of Normal Silica and NH_2-SAMMS for GI immobilization.

FIG. 17 shows a comparison of GI immobilization and pore size effect of NH_2-SAMMS.

FIG. 18 shows immobilization efficiency of GI in NH_2-SAMMS.

DETAILED DESCRIPTION OF THE INVENTION

A conceptual illustration of one embodiment of the protein system 2 of the present invention is shown in Fig. 1. A matrix material 4 has pores 6 containing protein 8. The protein 8 is connected to the matrix via connecting moieties 10. Many variations of this structure are possible. For example, while the figure illustrates a single protein in each pore, in many embodiments some pores will contain multiple proteins while other pores contain none. The present invention is not limited to the embodiment illustrated in Fig. 1.

The porous matrix material preferably has a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, more preferably, 100

to 200, and still more preferably 100 to 120 Å. For purposes of the present invention, pore size distribution is measured by N_2 adsorption using techniques that are well-known in the art. For materials with especially large pores, N_2 adsorption may need to be supplemented by mercury porisimetry or microscopy to get an accurate pore size distribution. As is conventional, "pore size" refers to pore diameter. In the protein system, the pore size distribution is to be measured without protein in the matrix - for measurement purposes, protein can be removed from the matrix by proteases or other appropriate means. For measurements on protein systems, the coupling agents remain bound to the matrix during measurement of pore size distribution. For purposes of characterizing methods of the present invention, or, of characterizing protein systems according to the method of making them, the pore size distribution of the porous matrix material is measured without coupling agents. The composition of the matrix material can vary, but is preferably an inorganic-oxide-containing material. Inorganic oxide based materials (such as silica-based materials) offer advantages over many organic supports - these advantages can include mechanical strength and chemical and thermal stability.

In preferred embodiments, the protein system comprises a coupling agent disposed between the inorganic porous matrix and the protein. The unreacted (that is, before reacting with a protein) inorganic oxide support typically has surface hydroxyl groups. Preferably, these surface hydroxyls are reacted with relatively low molecular weight organic compounds to form a functionalized monolayer. Treatment with the appropriate coupling agent can produce selected functionalizing moieties on the surface of the porous support. Preferred coupling moieties are mercapto (-SH), amino ($-NH_2$), carboxyl (-COOH), hydroxyl (-OH), and azido ($-N_3$). A particularly preferred embodiment utilizes the functionalized mesoporous support described by Feng et al. in "Functionalized Monolayers on Ordered Mesoporous Supports," Science, vol. 276, 923-926 (1997). As described in the article by Feng et al., the surface hydroxyls can be reacted with mercaptopropyltrimethoxysilane, $(MeO)_3Si(CH_2)_3SH$, to form a functionalized surface with terminal mercapto groups. Functionalized surfaces are superior to the nonfunctionalized surfaces because they provide better and more controllable chemical environments and bonding to proteins.

Where the surface of the porous matrix material is functionalized, it has been found that the degree of functionalization (as measured by surface coverage, where surface coverage is determined by transmission electron microscopy as described in the above-mentioned article by Feng et al.) effects the activity level of the bonded protein. Preferably, surface coverage is at least 2%, more preferably between about 20 and 70%, more preferably, between 20 and 50 %. Too many coupling moieties can reduce activity while too few reduces covalent attachment of the protein to the matrix and can reduce the stability of the protein system.

It has been found, especially for embodiments in which a coupling agent isn't used, that numerous factors can effect the porous matrix's ability to entrap proteins. A non-exhaustive list of these factors can include: the degree of functionalization, the type of functionalizing moieties (for example, amino, carboxyl, etc.), pH, ionic strength, buffering, and pore size. In general, electrostatic interactions can be very important for controlling entrapment of the protein. For example, carboxyls in the pores of the matrix material can interact with aminos on the surface of the protein, and vice versa. Similarly, hydrophilic/hydrophobic interactions can be controlled to tailor a matrix material for entrapping proteins; for example, hydrocarbon chains in the pores of the matrix material can interact with hydrophobic regions on proteins. Identifying the conditions for entrapping a selected protein can be determined through routine experimentation in view of the descriptions set forth herein.

Proteins are polymeric organic compounds comprising more than about 100 amino acid residues, and typically having molecular weights in the range of about 8,000 to about 300,000 daltons. Of most interest in the present invention are chemically active proteins, that is, those proteins that are capable of facilitating a chemical process such as hydrolysis, oxidation, reduction, oxygen transport, optical inversion, dehydrogenation, elimination, etc. More preferred are enzymes, that is, those proteins that catalyze chemical reactions. One particularly preferred protein is organophosphorus hydrolase (OPH) which is known and has been reported in the literature, see, for example, Muchandani et al., "Biosensor for direct determination of organophosphate nerve agents. Potentiometric enzyme electrode," Biosensors & Bioelectronics, 14, 77-85 (1999).

A protein can be comprised of amino acids that are all connected through covalent bonds. Proteins can also be comprised of subunits that are held together by non-covalent interactions. For example, hemoglobin is a protein that is comprised of four subunits. Proteins can also include other components such as metal atoms, porphyrin rings, and other manmade or naturally occurring modifications. OPH is a dimeric enzyme that has a diameter of about 45 to 80Å with a volume of about 1.95×10^5 Å^3. Thus, if a protein system were designed such that OPH occupied 10% of the average pore volume, the matrix would have an average pore volume of about 1.95×10^6 Å^3.

Protein size in the present invention is defined in the conventional sense based on the radius of gyration in the non-denatured state. In the protein systems of the present invention, a preferred type of proteins are enzymes having volumes in the range of 0.5×10^5 Å^3 to 3×10^5 Å^3, because proteins within this size (volume) range are especially advantageous in the porous matrices of the protein systems of the present invention.

The protein in the matrix can be compared to the protein in the "active state." In the present invention, the definition of activity (or "unit activity") for an immobilized protein is the

same as the accepted definition for the non-immobilized protein. Activity units are defined in terms of the quantity of protein required to produce a product from a known or characterized substrate in certain buffer conditions at a certain temperature for a specified time. For many enzymes and classes of enzymes, there are commonly accepted activity units. One source of commonly accepted activity units is the Worthington Enzyme Manual (available from the Worthington Biochemical Corporation, Freehold, NJ). This Manual contains definitions for the activity of enzymes including glucose oxidase (GOD) and glucose isomerase (GI). In the present invention, the activity of OPH is defined as described in Dumas et al., J. Biol. Chem., v. 264, p19659 (1989); an activity unit is the hydrolysis of 1 micromole of paraoxon per minute at 25 °C in 100 mM CHES at pH 9, typically monitored the change in absorbance at 400 nm when the paraoxon substrate is hydrolyzed to diethyl phosphate and p-nitrophenolate anion assuming the extinction coefficient, $\epsilon_{405} = 17{,}000\ M^{-1}cm^{-1}$. In preferred embodiments, the protein of the invention is at least 50% of the activity in the active state, more preferably at least 75%. We have found that activity of the OPH-containing protein systems have excellent activity. In some preferred embodiments, proteins, in systems of the present invention, have activities of 65 to 95 %. In some embodiments, the protein system of the present invention can also be defined in comparison to an equal mass of a system prepared and tested under the same conditions, including the same functionalization conditions, except using a normal silica as the porous matrix material; preferably the protein system has at least 2 times (2X), more preferably at least 5X, still more preferably at least 10X, and still more preferably at least 50X greater activity as compared to a system that is prepared identically except having a normal silica in the porous matrix material. The invention can also be defined as being able to maintain activity under the same conditions and to about the same or greater extent as described in the examples.

A nonlimiting list of proteins that may be employed in various embodiments of the invention is as follows: Adenosine aminohydrolase, Adenosine deaminase, Alcohol oxidase, Alcohol dehydrogenase, Amino acid oxidases, Amino acid deaminases, Amino acid decarboxylases, ATP kinase, ATP phophatase, Bilirubin oxidase, Catalase, Chymotrypsin, Creatine phosphokinase, Catecholase, Cholesterol oxidase, Chitinase, Chitodextrinase, Chitosanase, Cholesterol dehydrogenase, Cholesterol esterase, Choline oxidase, Choline phophatase, Citrase, Cocaine esterase, Cytochrome C, Cytochrome C oxidase, Cytochrome C reductase, Cytochrome P450, Cytochrome P450 reductase, Cytochrome B_5, DNase, DNA gyrase, helicase, DNA joinase, ligase, nucleotidylexotransferase, DNA nucleotidyltransferase, DNA polymerase, DNA repair enzyme, DNA topoisomerase, Endopectin lyase, Endopeptidase, Endoperoxide isomerase, Endoproteinase, Endoribonuclease, Fibrinogen, Ferredoxin, Ferredoxin reductase, Ferredoxin oxidoreductase, Ferroxidase, Galactose oxidase, Galactose dehyrogenase, GI, Glucose dehydrogenase, Glucose-6-phosphatase, Glucose-6-phophate dehydrogenase,

Glutathinone peroxidase, Glutathinone reductase, Hemoglobin, Heparinase, Lactose synthase, Lactoperoxidase, Lactic acid dehydrogenase, Lactate oxidase, lactase, laccase, Myoglobin, Malate dehydrogenase, Malic dehydrogenase, Malic enzyme, Methanol oxidase, Microperoxidase, Monophenol monooxygenase, Monophenol oxidase, Mutarotase, NADH dehyrogenase, oxidase, peroxidase, NADP cytochrome reductase, NADPH oxidoreductase, Nuclease, Nitrate Reductase, OPH, Oxalate decarboxylase, Oxalate oxidase, Peroxidases, Protease, Proteinase, Putidaredoxin, Papain, Pepsin, Plasmin, Plasminogen, Pectinase, Pyruvate carboxylase, decarboxylase, Puruvate dehydrogenase, oxidase, kinase, Quinone reductase, Ribonuclease, Salicylate hydroxylase, monooxygenase, Sarcosine dehydroxylase, oxidase, Sulfatase, Sulfite oxidase, Superoxide dismutase, Streptolysin O, Trypsin, Urate oxidase, Urease, Uricase, Xanthine dehydrogenase, oxidase, Actin, Beta Agarase, Albumin, Bovine Serum, Aldolase, Amylase, Alpha Amylase, Beta Aspartyl Aminotransferase, Avidin, Carbonic Anhydrase, Carboxypeptidase A, Carboxypeptidase B, Carboxypeptidase Y, Casein, Alpha Cellulase, Cholesterol Esterase, Cholinesterase, Acetyl Cholinesterase, Butyryl, Chymotrypsin, Clostripain, Collagen, Collagenase, Concanavalin A, Creatine Kinase, Deoxyribonuclease I, Deoxyribonuclease II, T4 DNA Ligase, DNA Polymerase I, T4 DNA Polymerase, Dextranase, Diaphorase, Elastase, Elastin, Galactosidase, Beta Glucose-6-Phosphate Dehydrogenase, Beta Glucosidase, Beta Glucuronidase, Glutamate Decarboxylase, Glyceraldehyde-3-Phosphate Dehydrogenase, Glycerol Dehydrogenase, Glycerol Kinase, Hexokinase, Hyaluronidase, Hydroxysteroid Dehydrogenase, Leucine Aminopeptidase, Lipase, Luciferase, Lysozyme, Malate Dehydrogenase, Maltase, Mucin, NADase, Neuraminidase, Micrococcal Nuclease, S1 Nuclease, Ovalbumin, Oxalate Decarboxylase, Pectinase, Acid Phosphatase, Alkaline Phosphatase, Phosphodiesterase I, Phosphodiesterase II, Phosphoenolpyruvate Carboxylase, Phosphoglucomutase, Phospholipase A2, Phospholipase C, Plasma Amine Oxidase, Pokeweed Antiviral Toxin, T4 Polynucleotide Kinase, Polyphenol Oxidase, Protease, S. aureus, Proteinase K, Pyruvate Kinase, Reverse Transcriptase, Ribonuclease, Ribonuclease T1, Ribonucleic Acid, RNA Polymerase, RNA Polymerase, T7, and Xylose isomerase.

In various embodiments, the invention can be defined as including any of the proteins listed above (either exclusively, substantially, or including). For example, the invention can be defined wherein the protein is exclusively (i.e., consists of), or substantially (i.e., consists essentially of), or includes (i.e., comprising) OPH (or, alternatively, any of the other proteins from the above list).

While there is an enormous variety of proteins, there is also an enormous overlap in the chemical moieties that make up the protein structure. The same types of amino acids are common to most proteins. This similarity in chemical moieties enables the same coupling techniques to be used to bond proteins onto supports. For example, the sulfhydryl of cysteines,

the amino and carboxyl-terminal amino acids, and the amino groups of arginine and lysine, regardless of the protein in which these moieties reside, can typically be similarly reacted with coupling agents or the matrix surface.

In most instances, the protein is not directly bonded to the support. In some cases, a connecting moiety or moieties bonds to the support and the protein. For example, the coupling agent can be reacted via hydroxyl moieties on the support with amines on the protein (see, for example, U.S. Patent No. 5,077,210 which is incorporated herein by reference). These connecting moieties are preferably organic moieties having a chain length of 2 to 20 atoms, more preferably 4 to 10 atoms. Preferably, each protein is bound to the matrix via at least one coupling moiety, more preferably via 2 to 10 moieties. The number of moieties bound to each protein can be determined by appropriate analytical techniques, for example, by cleaving off the bound proteins and analyzing the cleaved molecules by mass spectrometry. There are a large number of known coupling agents for connecting surface hydroxyls to proteins. For example, a coupling agent can have a siloxane ($-Si(OR)_x$) terminal group that forms oxo bonds to the surface, a flexible organic chain (e.g., $(CH_2)_n$), and a thiol (-SH) terminal group that bonds with a protein.

The protein system combining the support and the entrapped (for example, the non-covalently bonded, or covalently attached) protein can be difficult to characterize with chemical precision. However, the system can be characterized by measurable properties. Measurable properties that can define various embodiments of the invention include: pore size, pore volume, pore size distribution, surface area, activity, stability, density of protein in support, density of system, and strength of system. It has been discovered that superior properties can be obtained by engineering supports with pore sizes (or pore volumes) that correspond to protein sizes (or protein volumes). Preferably the volume of a protein is between 5 and 40 % of the average pore volume (where, for purposes of this metric, the average pore volume is based only on those pores in the size range of 50 to 400Å), more preferably the volume of a protein is between 10 and 25 % of the average pore volume. This size matching of protein to pore size can produce surprising improvements in activity and stability. Although the mechanisms causing these improved properties are not fully elucidated, it is believed that the confinement of the protein may help to direct reactive species into the protein and may prevent the protein from irreversibly unfolding. Protein volume can be measured by biophysical methods such as analytical ultracentrifugation or x-ray crystallography. Preferably, the activity, measured per protein molecule, is at least 60% of the protein's activity in the active state. Preferably, the system comprises less than 40 volume % protein; more preferably 5 to 40 volume % protein; still more preferably 10 to 25 volume % protein. Preferably, the system possesses stability such that when exposed to 8M urea (as set forth in the examples), the protein in the protein system is at least twice as stable as the free protein, in some embodiments about 3 to about 5 times more stable. In other embodiments, the

protein in the protein system is at least twice as stable (as measured by the rate of denaturing), more preferably at least 3 times as stable, as the free protein when placed in solution under any of the conditions that are set forth in the Examples.

Another advantage obtainable by the present invention is high surface area, as measured by N_2 adsorption, of the protein system. As with pore size, surface area is measured on the functionalized surface for protein systems and on the unfunctionalized matrix material for the invention defined by methods and systems made by these methods. Surface area is preferably at least 700 m^2/g; more preferably at least 900 m^2/g. The upper limit of surface area may be limited by the upper limit of the mesoporous matrix materials of the type described by Feng et al. and similar materials. Another advantage of the present invention is that it can produce a relatively dense protein system. Preferably, the protein in the system has a density of at least 0.01 mmol/g; more preferably a density of 0.1 to 1 mmol/g. The inventive systems can be characterized by exhibiting any one of its properties or several of its properties in various combinations. For example, in a preferred embodiment the protein system exhibits an activity of 65 to 95% that of the active protein and has a density of 0.1 to 1 mmol/g.

In some preferred embodiments, the porous matrix material, including entrapped protein, is disposed within a microchannel. A microchannel is a channel that has at least one dimension of 2 mm or less, preferably 1 mm or less. Microchannels can offer advantages such as enhanced heat and/or mass transfer and compactness of design. Testing with OPH cross-linked into SAMMS disposed in a 0.25 mm wide channel has demonstrated low pressure drop (flow under gravity or peristaltic pumping or less than 1 bar) and fast reaction times. Thus, the invention includes apparatus and methods in which a porous matrix material, including entrapped protein, is disposed within a microchannel. In the methods, for example, a reactant can be passed through the microchannel to form products.

Proteins can be prepared by known procedures and, in preferred embodiments, do not need special procedures before reaction with coupling agent(s) to bond to the support. Preferably, prior to bonding within the matrix, the protein should be about 95% pure in an aqueous solution that stabilizes activity, and the buffer should not hinder the coupling chemistry.

In the inventive method of preparing OPH, a host cell is transfected with a vector comprising a sequence encoding OPH, the sequence being operably linked to a T7 expression control sequence. The transfected host cell is cultured under conditions permitting expression under the control of the expression control sequence. The OPH is purified from the cell or the medium of the cell. In preferred embodiments, the vector is provided with the sequence encoding OPH operably linked to the T7 expression control sequence. Preferably, the OPH has an activity of about 13,000 units/mg. Preferably, the vector is a plasmid. The host cell can be a

prokaryotic cell, eukaryotic cell, or yeast cell. The prokaryotic cell is preferably a bacterium, more preferably the bacterium is *Escherichia coli*. The yeast cell is preferably *Pichia pastoris*.

The matrix is preferably a mesoporous oxide material made from soluble precursors. Examples of preferred syntheses are provided in U.S. Patents Nos. 5,645,891 and 5,922,299 and U.S. patent application ser. no. 09/020,028, all three of which are incorporated herein as if reproduced in full below, Liu et al., "Molecular Assembly in Ordered Mesoporosity: A New Class of Highly Functional Nanoscale Materials," J. Phys. Chem., 104, 8328-8339 (Aug. 2000), and the Feng et al. article referenced above.

A typical synthesis for a matrix material was reported by Feng et al., Science, 276, p923 (1997). A CTAC/OH solution was prepared by contacting a CTAC solution with a strongly basic ion exchange resin (DOWEX-1, 0.2 g resin per gram of 29% CTAC solution). 13 g of colloidal silica, 51 g of tetramethylammonium silicate and 28 g of mesitylene were added to each 100 g of CTAH/OH solution. The mixture was sealed in a teflon™-lined vessel and heated at 105°C for 1 week. The product was recovered by suction filtration, dried at ambient temperature, and calcined at 540°C for 12 hours in air. The surface of the resulting mesoporous material was functionalized by a variety methods. For example, the surface can be functionalized with thiol groups by reaction with tris(methoxy)mercaptopropylsilane. The resulting functionalized matrix is called a "SAMMS." The percent surface coverage was estimated based on (i) the surface area of the support, (ii) the weight change after the functionalized monolayer was attached, and (3) the ideal loading density that could be achieved on flat surfaces. The percent surface coverage can be verified by electron energy-dispersive spectroscopy (EDS).

As known in the art, various approaches can be used to attach a protein for a support. In a preferred embodiment, the support is pretreated with a coupling agent, such as bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl]sulfone (BSOCOES). Excess coupling agent can be washed out. A protein is subsequently reacted with the coupling-agent-treated surface. Alternatively, protein can first be reacted with the coupling agent, and subsequently reacted with the surface of the matrix. Excess protein can be washed out and recovered.

In a preferred method, the functionalized porous matrix is first reacted with a cross-linking agent. For example, an amino-derivatized SAMMS is reacted with sulfo-BSOCOES or glutaric dialdehyde. If present, it is desirable that excess cross-linking agent is removed. Then, a protein is added. Preferably, the protein is in a low concentration solution. This procedure reduces or eliminates the possibility intermolecular cross-linking of the protein by the cross-linking agent.

In an alternative method, a porous matrix material (preferably a functionalized porous matrix material) is combined with a protein, without the addition of a cross-linking agent. In this manner, a protein system can be prepared in which the protein is noncovalently bonded to a

porous matrix. This method is preferably conducted by tailoring electrostatic interactions such that the porous matrix creates a complementary environment for the selected protein (for example, amino groups in the pores of the matrix match up with carboxyl groups on the protein). Factors that may be considered in tailoring the electrostatic interactions have been discussed above.

Protein systems, as described herein, can be made using any of the methods described herein, and these methods are also part of the present invention.

EXAMPLES

We obtained the OPH gene available from the ATCC and sub-cloned it into 2 vectors purchased from Novagen, pET11a and pET15b. Two Novagen vectors were used so that both a native version of OPH and an OPH containing a His-Tag™ could be produced. We produced multiple clones of both types. These Novagen vectors contain strong promoters and they are designed to maximize desired protein yields. Restriction digests confirmed that we correctly sub-cloned the OPH gene and the resulting constructs yielded active OPH protein: SEQ ID No. 4 (Fig. 4).

Bacterial expression and purification: After the recombinant OPH protein was linked to the nanoporous substrate, purification steps were carried out. The total expression levels achieved were on the order of 4 g/liter for total protein. We purified ~10 mg/liter active protein from the soluble fraction. Thus, most of the OPH is in inclusion bodies; *i.e.* it is present in an inactive form. The fact that the protein is present in inclusion bodies simplifies purification. OPH purified directly from washed and centrifuged inclusion bodies appears almost as pure as OPH purified by affinity column chromatography as analyzed by SDS polyacrylamide gel electrophoresis. Large scale methods for recovering activity from inclusion body proteins may be developed through routine experimentation. This simplified purification procedure is suitable for industrial production.

MATERIALS AND METHODS

Materials:

Diethyl p-nitrophenyl phosphate (paraoxon, 90%), different metals salts, glycerol and all buffers and other salts were purchased from Sigma®-Aldrich®.

Components of fermentor media (Peptone and Yeast extract) were obtained from Gibco BRL, expression vectors (pET11a™, pET15b™) were purchased from Novagen Inc., Madison, WI. Primers for PCR were ordered from Genosys Inc..

Bulk chromatography media for the protein purification was obtained from Perseptive Biosystems (HS™ and HQ™).

Polypropyl A™ columns as well as nonderivatized silica resin for comparison of OPH linking obtained from Poly LC Inc., Columbia, MD.

Cross-linking reagents for enzyme immobilization were purchased from Pierce Chemical Company, Rockford, IL.

The abbreviations used:

CTAC, cetyltrimethylammonium chloride

OPH, organophosphorous hydrolase,

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid,

CHES, 2-(cyclohexylamino)ethanesulfonic acid,

IPTG, isopropylthiogalactoside,

SAMMS, self-assembled monolayers on mesoporous silica,

Sulfo-BSOCOES, bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl]sulfone,

DTSSP, dithiobis(sulfosuccinimidylpropionate).

β-ME, β-mercaptoethanol

Matrix Synthesis:

A typical procedure for preparing the mesoporous oxide material used in the Examples is as follows. Mesoporous silica with 300Å pore diameter was prepared by a liquid crystal templating procedure. Triblock copolymer, Pluronic P123 (a propylene oxide/ethylene oxide copolymer available from BASF, M_{av} = 5,600) was used as a structure-directing agent and mesitylene as a pore expending agent. 20g of Pluronic P123 was dissolved in 150 g of deionized (DI) water and 600g of 2 M HCl solution at 40°C with stirring. 31.9g of mesitylene was then added and kept stirring at the same temperature. 42.5g of TEOS was added dropwise into the cloudy micelle solution and cured at that temperature for 20 h with stirring. The mixture was aged in a teflon-lined autoclave at 100°C overnight without stirring. The white solid was filtered, washed with DI water, and air-dried. This solid was calcined at 550°C for 6h with slow increasing temperature (1°C/min).

In a typical preparation of 20% propylcarboxylic acid functionalized mesoporous silica, 2.0g of mesoporous silica (average pore size = 30 nm, surface area = 533 m^2/g) was first suspended in toluene (60mL) and pretreated with approximately a bilayers' worth of DI water (0.64ml). This suspension was stirred for 2 hours to distribute the water throughout the mesoporous matrix. The hydrated mixture was then treated with 20% (0.288g) of 1 monolayer's 3-cyanopropyltrimethoxysilane (CPTS) and heated to reflux for 6 hours. The treated mesoporous

silica was washed with toluene to remove any unreacted silanes. The air-dried CPTS-SAMMS materials were then treated with 50% H_2SO_4 solution and refluxed for 3 hours. After washed with DI water extensively, the white sample was dried under vacuum at 70°C overnight.

In case of 20% aminopropyl (APTS) and mercaptopropyl (MPTS) functionalized silica, the same procedure was applied without hydrolysis step. To the suspension of 2.0g of mesoporous silica, toluene (60mL) and 0.64g of water and 0.188g of APTS or 0. 206g of MPTS were added separately. The mixtures were heated to reflux for 6h, and then filtered off, washed with ethanol, and dried under vacuum at 70°C overnight.

OPH subcloning:

OPH sequence was cloned by PCR using pCMS75 plasmid in E.coli FM5 (Amgen Inc.) which had been obtained from American Type Culture Collection, Rockville, MD (ATCC® #67778). Primers for PCR reaction were chosen using Primer Premier™ software, version 4.04, from Premier Biosoft International. The primers used were listed as following:

OPH upstream primer, 26-mer: SEQ ID No. 1 5' TAAATTATCTCTGGCGGTGTTGACAT 3'

OPH downstream primer with BamHI restriction site (recognition sequence in bold), 20-mer: SEQ ID No. 2 5' GAAGGATCCAGATGGCGTCA 3'

OPH sequence was subcloned using NdeI, BamHI restriction sites into pET11a. The resulting OPH sequence encoded the mature portion of OPH enzyme, i.e., without N-terminal 29 amino acid signal sequence, so that the length of the sequence is 1010 bp, which corresponds to 337 amino acids in total or 36,419 Da in MW.

The confirmation of the correct product of cloning was made by PCR and restriction enzyme digest.

A plasmid diagram for OPH is illustrated in Fig. 2.

Expression and purification:

The organophosphorous hydrolase was purified from E.coli expression system using oph-pET11a plasmid and BL21(DE3) pLyss™, Novagen Inc., as a host strain. (N-terminal His-Tag™ OPH subcloned into pET-15b plasmid) Induction with IPTG was shown to produce a protein which has apparent mobility on SDS gel corresponding to prediction based on the gene sequence of mature native OPH protein without N-terminal signal sequence (about 36 kDa or with the N-terminal His-Tag about 38 kDa). Identity of the recombinant product was also confirmed by aminoacid analysis and appearance of Paraoxon hydrolysis activity in crude cells lysate after induction with IPTG.

The general protocol used for propagation of cells in Bio-Flo 3'000 fermenter™ (New Brunswick, Inc.) was as follows:

The E.coli cells were grown for 12 hours at 30 °C in a flask, 100 µl of glycerol stock/1L of LB media, 100 µg/ml ampicillin, 35 µg/ml chloramphenicol, and this starting culture was used as inoculums for the fermenter. 500 ml ON culture with OD=0.5 were spun down, washed with fresh LB media, spun again, redissolved in 250 ml of LB without antibiotics, added to the fermenter media, total volume=2.5 L. (NOTE: make sure not to add 1mM $CoCl_2$ to LB media for ON starting culture, since it kills the cells).

Cells in the fermenter reached mid-log phase after 4 hrs at 37 °C in a medium containing 5 g/l Yeast Extract, 10g/l Peptone, 5 g/l NaCl, 1 ml/l antifoam, 60 mM K_2HPO_4, 15 mM KH_2PO_4, 1 mM $CoCl_2$, 1.32 µg/ml thiamine, 100 µg/ml ampicillin, 35 ug/ml chloramphenicol, 10 g/l glycerol and trace metals (10 µM $NH_4Mo_7O_{24}$, $CuSO_4$, H_3BO_3, $MnCl_2$, $ZnCl_2$), 50 µM $FeCl_3$, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$. Oxygen level was maintained at 35% using DO-agitation-oxygen triple cascade, setting up agitation range 200 rpm minimum to 800 rpm maximum. Initial glucose concentration in the media was 10 g/l, glucose level was monitored during the run using regular glucose strips and kept to be not less than 2 g/l.

When agitation reached 467 rpm, OD550=5, glucose level was 2 g/l. Thirty-one (31) ml of 40 % glucose were added to bring the glucose level to 5 g/l. When agitation reached 700 rpm, OD550=15, glucose level dropped again to 2 g/l. The temperature was lowered to 28 °C, the mixture was induced with 0.25 mM IPTG and another 31 ml of 40 % glucose were added. After 4 hrs of induction at 28 °C, another 0.25 mM IPTG were added (total of 0.5 mM). The glucose level was 2 g/l again and 63 ml of 40 % glucose were added to bring the glucose level to 10 g/l. After 2 more hours of induction, cells obviously continued to grow, temperature was lowered to 24 °C and the cells were left in a fermenter ON for another 14 hrs. Finally, the cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C.

From 2.5 l of cell culture, about 150 g of wet weight cell paste was isolated and the cell paste was stored at –80 °C.

We were able to purify from 60 g of cells (corresponds to 1 L of culture) about 90 mg of OPH with activity of 13,294.12 units/mg. This yield can be compared to the literature. Omburo G.A., Kuo J.M., Mullins L.S., and Raushel F.M., in Characterization of the Zinc Binding site of Bacterial Phosphotriesterase. JBC, 1992, v.267(5): 13278 – 13283 reported getting from 160 g of cells about 298 mg of cobalt phosphotriesterase with activity 8'020 units/mg.

Lai K., Dave K.I., and Wild J.R. (Bimetallic Binding Motifs in Organophosphorous Hydrolase Are Important for Catalysis and Structural Organization. JBC, 1994, 269(24): 16579 – 16584), which is more difficult to compare, reported purifying 5 mg of OPH per 1 L of culture (probably grown in flasks). They favored using weak promoters for expression (native Plac) versus strong promoters, like T7, because the yield of OPH activity they got with strong promoter constructs

was lower (data not shown). All purification steps were performed at 4 °C using pre-cooled equipment and Revco Chromatography Refrigeration cabinets.

The bacterial cells (60 g) were suspended in 420 ml of lysis buffer A, containing 100 mM HEPES pH 8.5, 50 uM $CoCl_2$, 1 mM DTT, antiprotease cocktail (pepstatin, leupeptin and aprotinin), and cells were lysed using French pressure cell 2 times. Soluble protein supernatant obtained by 100,000 x g centrifugation for 1 hr (Avanti™, Bechman), was loaded on 500 ml HQ™ anion-exchange column (Perseptive Biosystems) equilibrated in buffer A, with substitution of 1mM DTT for 5 mM beta-mercaptoethanol (2-me). The column flow rate was 25 ml/min. Flow-through containing OPH was collected, pH was adjusted to 7.5 using 1 M MES, pH 5.5, and applied on a 250 ml HS cation exchange column (Perspective Biosystems), equilibrated in 0.1 M HEPES, pH 7.5, 50 uM $CoCl_2$, 5 mM 2-me. The column flow rate was 25 ml/min. Flow-through of HS column was retained, enough dry $(NH_4)SO_4$ was added to make final conductivity of the sample to be equal to conductivity of 1 M $(NH_4)SO_4$ solution (i.e., 105 mS /ml), using a conductivity meter Orion 126, Cell 012210. A sample was loaded on a 180 ml Polypropyl A™ (PolyLC, Inc.) column, equilibrated in 1 M $(NH_4)SO_4$, 0.1 M HEPES pH 7.5, 5 mM 2-me. The column flow rate was 10 ml/min.

After 3 column volume wash with equilibration buffer 10 column volume gradient to 0.1 M HEPES pH 7.5 was applied, OPH was eluted in the very end of the gradient. We were able to purify close to 100 mg of pure OPH from 60 g of cells (corresponds to 1 L of culture). After concentrating the protein up to 3 mg/ml using Milipore UltraFree ®Biomax centrifugal concentrators with 30 K NMWL (30 kDa cut-off) membrane and dialysis against 20 % glycerol to 0.1 M HEPES pH 7.5 50 uM $CoCl_2$ protein was aliquoted and stored at –80 °C. Specific activity was determined as 13,294.12 units/mg (see Table I and summary SDS gel).

The difference in activity of the protein which came as a peak from HIC and the same protein after concentration and dialysis probably may be explained by the buffer exchange. In one case, the buffer contained beta-mercaptoethanol, the competitive inhibitor of OPH activity, and no $CoCl_2$, the other the buffer contained 50 μM $CoCl_2$ and no 2-me.

Table I

Purification of OPH (Co in active center)

Purification stage	Total protein, mgs	Volume, ml	Total activity, units	Purification, fold	Specific activity units
Cell lysate 100k supernatant 62 g cells	20,925	620	1,133,364 (100%)	1.0	54.16
HQ 500 ml flow-through (HS starting material)	10,044	1000	923,435 (81.48 % recovery)	1.69	91.94
HS 250 ml flow-through (HIC starting material)	7,714	1240	903,529 (79.72 % recovery)	2.16	117.13
HIC 180 ml OPH peak	100	180	468,465 (41.33 % recovery)	86.5	4,684.66
Pure OPH after concentration and dialysis, 3 mg/ml	90	28	1,196,473	not applicable	13,294.1

OPH immobilization:

Media used:

1. SAMMS: derivatized with SH-, COO-, NH_2- active groups, 5 % and 20 % coating (5 % and 20 % of all available silane groups get modified or derivatized with active groups). Characteristics of the media: 250 Å 12 – 15 um beads, surface area around 450 m^2/g

2. Poly LC Silica: Purchased uncoated, derivatized in PNNL with NH_2-, COO- groups, 20 % and 100 % coating. Characteristics of the media: 300Å 12 um beads, surface area around 100 m^2/g.

After screening for the best linking chemistry that would give the highest density of bound enzyme as well as lowest losses of activity and lowest diffusion limits, we chose linking of OPH through its NH_2- groups to NH_2- derivatized media.

Many cross-linking agents were tested, among them 2 were found to be especially efficient: Sulfo-BSOCOES, bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl]sulfone, and DTSSP, dithiobis(sulfosuccinimidylpropionate). The advantages of these 2 cross-linking agents are as follows:

i. Both cross-linking agents have spacer arms (12 Å in length for DTSSP and 13 Å for BSOCOES). A spacer arm is beneficial to avoid steric hindrance.

ii. Both of them are water soluble due to the sulfo- functioning group.

iii. Pierce (Rockford, IL) recommended PBS as the linking buffer, pH 7.5. This pH is favorable for OPH because OPH tends to aggregate and lose the metal from active center at an acidic pH, i.e., pH lower than 6.5. A higher pH has another advantage:

The hydrolysis of NHS-esters proceeds faster at a higher pH. Therefore when we used a higher pH, we obtained a higher molar ratio of cross-linking agent per protein.

iv. Both produce stable covalent amide bond, which in case of DTSSP is cleavable with thiols (DTT, mercaptoethanol, etc.). This feature could be useful for certain applications.

Standard protocol for sulfo-BSOCOES or DTSSP coupling of OPH to NH_2-derivatized surface:
(Pierce protocol with little modifications)

1. Sulfo-BSOCOES or DTSSP cross-linking reagents (come in tubes) should be stored at –20°C, preferably desiccated, under nitrogen. In practice it is a good idea to use nitrogen glove box or nitrogen bags (filled with nitrogen using a nitrogen tank in a cold room) when work with the cross-linking reagents (i.e. to aliquot the content of the original tube). Always let the reagents come to room temperature before opening the tube.
2. The media that is planned to link OPH to should be well swollen in water. In general we used 500 mg of media / 5 ml H_2O and made a ~50 % slurry (v/v) for SAMMS. This slurry was very stable when stored at +4 °C. NOTE: For PolyLC media: 2x more media in dry weight compared to SAMMS should be used (i.e. ~ 1g of PolyLC media / 5 ml H_2O and make a ~50 % slurry (v/v)).
3. OPH, 09/02 purification, [3 mg/ml], aliquoted in 2 mls, stored at –80°C, was thawed, and the buffer was changed from 25 mM HEPES pH 8.5, 20 % glycerol, 50 uM $CoCl_2$ to 0.1 M carb/bicarbonate, pH 9.0. The NAP-25 column that we used had a max volume of 10 ml. With that, we were able to apply about 2.5 – 3.0 ml of the max sample volume.
4. Concentrate up to 20 mg/ml using the new Millipore Biomax Ultrafree 4.0 ml 30K CO membrane unit, for 10' at +4°C Sorvall CF, bucker rotor, at max speed.
5. With a cut yellow tip, added 50 % slurry powder in H_2O to OPH, in 0.1 M carb/bicarbonate buffer pH 9.0, 90.1 ml volume for 2 mgs. The approximate ratio for the slurry powder is 150 ul 50 % slurry / 2 mg of protein: 2 types of derivatization (NH_2- 20 % and 100 % coverage) of PolyLC silica, and NH_2-SAMMS 20 % coverage.
6. Dissolve 1.5 mg or 3 mg or 6 mg Sulfo-BSOCOES or DTSSP in 590 ul of 5 mM MES pH 5. (to get 10x, 25x, and 50x molar ratio of cross-linking agent/protein)
7. Immediately add the cross-linking agent solution (125 ul /2 mg of protein) to each eppendorf tube drop-wise, mixed, put the tube on rotating device, at room temperature for 45 min.
8. Add stop-solution : 1 ml of 1 M Tris pH 8.1, 1 hr at room temperature.

9. Wash with PBS 2 times, 0.5 M NaCl-PBS one time, and PBS one time. Re-suspend in 100 mM HEPES pH 8.5 – 50 uM $CoCl_2$ and stored at +4°C.

10. Estimate the amount of protein lined to the resin using BCA Pierce kit, allow to react at 37 °C for 30 min.

Example 1

Coupling of OPH to SAMMS-NH_2 derivatized surface using Sulfo- BSOCOES 20 % coating, with 50x molar excess of cross-linking agent per protein.

The standard protocol was followed, thus:

1. A nitrogen glove box filled with nitrogen was used for taking aliquots of the content out of the tubes containing Sulfo-BSOCOES.

2. Five hundred (500)mg of media were used in 5 ml H_2O and made a ~50 % slurry (v/v). This slurry was very stable when stored at +4 °C.

3. OPH, 09/02 purification, [3 mg/ml], aliquoted in 2 ml, stored at –80°C, was thawed, and the buffer was changed from 25 mM HEPES pH 8.5, 20 % glycerol, 50 uM $CoCl_2$ to 0.1 M carb/bicarbonate, pH 9.0 using Pharmacia Sephadex G-25 column. The NAP-25 column that we used had a max volume of 10 ml. With that, we were able to apply about 2.5 – 3.0 ml of the max sample volume.

4. Concentrate up to 20 mg/ml using the new Millipore Biomax Ultrafree 4.0 ml 30K CO membrane unit, for 10 min at +4 °C Sorvall CF, bucker rotor, at max speed.

5. With a cut yellow tip, added 50 % slurry powder in H_2O to OPH, in 0.1 M carb/bicarbonate buffer pH 9.0, 90.1 ml volume for 2 mgs. The approximate ratio for the slurry powder is 150 ul 50 % slurry / 2 mg of protein: 2 types of derivatization (NH_2- 20 % and 100 % coverage) of PolyLC silica, and NH_2-SAMMS 20 % coverage.

6. Dissolve 6 mg of Sulfo-BSOCOES in 590 ul of 5 mM MES pH 5.

7. Immediately add the Sulfo-BSOCOES solution (125 ul /2 mg of protein) to the eppendorf tube drop-wise, mixed, put the tube on rotating device, at room temperature for 45 min.

8. Add 1 ml of 1 M Tris pH 8.1, 1 hr at room temperature.

9. Wash with PBS 2 times, 0.5 M NaCl-PBS one time, and PBS one time. Re-suspend in 100 mM HEPES pH 8.5 – 50 uM $CoCl_2$ and stored at +4°C.

10. Use a BCA Pierce kit, allow to react at 37°C, the estimated amount of OPH linked to the resin = 25.0 mg/ml media and 125.0 mg/g media.

Example 2

Coupling of OPH to SAMMS-NH_2 derivatized surface using Sulfo- BSOCOES 20 % coating, with 25x molar excess of cross-linking agent per protein. The procedures were similar to Example 1, except for step 6: 3 mg of Sulfo- BSOCOES were used instead. The estimated amount of OPH linked to the resin = 16.0 mg/ml media and 8.0 mg/g media.

Example 3

Coupling of OPH to SAMMS-NH_2 derivatized surface using Sulfo- BSOCOES 20 % coating, with 10x molar excess of cross-linking agent per protein. The procedures were similar to Example 1, except for step 6: 1.5 mg of Sulfo- BSOCOES were used instead. The estimated amount of OPH linked to the resin = 5.0 mg/ml media and 25.0 mg/g media.

Example 4

Coupling of OPH to SAMMS-NH_2 derivatized surface using DTSSP 20 % coating, with 50x molar excess of cross-linking agent per protein. The procedures were similar to Example 1, except for step 6: 6 mg of DTSSP were used instead. The estimated amount of OPH linked to the resin = 25.0 mg/ml media and 125.0 mg/g media.

Example 5

Coupling of OPH to SAMMS-NH_2 derivatized surface using DTSSP 20 % coating, with 25x molar excess of cross-linking agent per protein. The procedures were similar to Example 1, except for step 6: 3 mg of DTSSP were used instead. The estimated amount of OPH linked to the resin = 16.0 mg/ml media and 80.0 mg/g media.

Example 6

Coupling of OPH to SAMMS-NH_2 derivatized surface using DTSSP 20 % coating, with 10x molar excess of cross-linking agent per protein. The procedures were similar to Example 1, except for step 6: 1.5 mg of DTSSP were used instead. The estimated amount of OPH linked to the resin = 5.0 mg/ml media and 25.0 mg/g media.

Example 7

OPH was coupled to PolyLC at 20 % and 100 % coating surface using Sulfo-BSOCOES and DTSSP, with 10x, 25x, and 50x molar excess of cross-linking agent per protein. The procedures were similar to Example 1 to 6, except for step 2: 1000 mg of PolyLC were used per 5 ml H_2O and made ~50 % slurries (v/v).

The estimated amount of OPH linked to the resin is listed in Table II.

Table II

Media	SAMMS-NH_2, 20% coating			PolyLC, 20% coating			PolyLC, 100% coating		
molar excess x-linking agent	10 x	25 x	50 x	10 x	25 x	50 x	10 x	25 x	50 x
OPH bound, mg/ml media	5.0	16.0	25.0	5.0	5.0	6.74	7.0	8.5	9.0
OPH bound, mg/g media	25.0	80.0	125.0	12.5	12.5	16.85	17.5	21.5	22.5

It can be seen that inventive compositions are capable of higher density loading than with conventional silica (PolyLC). Thus, preferred embodiments of the invention can be characterized by loading densities. Preferably the protein system has density that is 2 to about 7 times higher (in mg/g) than PolyLC with the same coating %, more preferably about 5 to about 7 times higher. In a preferred embodiment, the densities are measured at a 20% coating.

Example 8 - Effect of Denaturing

Stability to denaturing conditions of OPH-SAMMS and soluble OPH was conducted using urea as the denaturing agent at concentrations of 4 M, 6 M and 8 M. The phrases "Soluble OPH" or "OPH soluble" in the Examples section refers to the non-inclusion body OPH that was released during cell breakage with the French Press and soluble in the buffers indicated for each purification step. The results are shown in Table 3 below.

Table III

	4M		6M		8M	
OPH Soluble / immobilized	soluble	imoblized	soluble	imoblized	soluble	imoblized
Activity %	94 ± 8	108 ± 10	39.5 ± 0.3	72.1 ± 0.7	4.0 ± 0.5	21.6 ± 0.2

The immobilized enzyme was far more stable than the free protein. Thus, preferred embodiments of the invention can be characterized by their stability to denaturing agents. Preferably the protein system has a stability, in 8M urea, that is at least twice as stable as the free protein, and in some embodiments, about 3 to about 5 times more stable.

Example 9 - Recovery From Dehydration

Dehydration and recovery experiments were conducted using the immobilized OPH-SAMMS and soluble OPH. While soluble OPH retained only 7 ± 1 % of its activity, the OPH-SAMMS completely retained its activity (106 ± 8 %).

Example 10 - Kinetic Properties

A kinetic study was performed with immobilized OPH on SAMMS, immobilized OPH on PolyLC, and soluble OPH. Enzymatic activity was measured using 1 mM paraoxon solution at 25 C and monitoring the change in absorbance at 405 nm when substrate was hydrolyzed to diethyl phosphate and p-nitrophenolate (extinction coefficient 17,000 M^{-1} cm^{-1}) in 100 mM CHES buffer, pH 8.0, 50 uM $CoCl_2$. Analysis was with a Hewlett-Packard model 8453 UV/Vis spectrophotometer in kinetics mode equipped with the Thermostable Cell Holder and Cell Stirring Module. Fresh dilutions of substrate were prepared no more than 30 minutes before measurements. SigmaPlot was used to draw linear regressions of the data. The PolyLC (Columbia, MD) silica was purchased uncoated, and derivitized with amino groups. The pore size of the 12 um beads was 300 Å. Both OPH immobilized of SAMMS and PolyLC have the

same K_m as soluble OPH, but demonstrated lower V_{max}: 2.83 fold lower for OPH-SAMMS, and 6.44 fold for OPH-PolyLC. That is: OPH-SAMMS has approximately 2.3 times faster reaction rate than OPH-PolyLC. Mass in the table refers to mass of OPH.

	OPH soluble		OPH-SAMMS		OPH-PolyLC	
Concentration, µg/ml	0.150	0.375	0.16	0.40	0.15	0.40
K_m, mM	0.099	0.125	0.086	0.127	0.087	0.130
V_{max}, AU/s	0.50	1.87	0.15	0.66	0.08	0.29

Example 11 - Stability in Alkaline pH

In this experiment, OPH-SAMMS and soluble OPH were tested for stability under alkaline conditions. After one hour of alkaline pH treatment (1M Tris, pH 12.0), the OPH-SAMMS was found to retain 11.6% of its activity as compared with 0.77% for the soluble OPH, and after 24 hours of this alkaline treatment, the OPH-SAMMS was found to retain 9.9% of its activity as compared with 0.03% for the soluble OPH. The conditions in this example define what is meant by "alkaline conditions" as that term is used in this application. While the example illustrates an OPH system, it should be recognized that the stability advantages provided by the mesoporous matrices of the present invention are general, and it is expected that other proteins will obtain similar stability advantages.

Example 12 - Thermal Stability

Experiments studying thermal stability showed that OPH-SAMMS was significantly more stable than OPH in solution. The results of these experiments are shown in the Tables below.

	OPH soluble								
Storage Time, days	7			14			30		
Temperature, °C	4	room	37	4	room	37	4	room	37
Activity %	95	85	80	92	80	65	90	75	50

	OPH-SAMMS								
Storage Time, days	7			14			30		
Temperature, °C	4	room	37	4	room	37	4	room	37
Activity %	100	100	5	105	107	85	102	103	80

Example 13- Effect Of Lyophilization

OPH-SAMMS and soluble OPH were subjected to lyophilization conditions (1M MES, pH 5.0) and it was found that OPH-SAMMS retained 50% of its activity after 1 and 24 hours, while the soluble OPH retained only 15% of its activity after 1 and 24 hours.

Example 14

(1) A nitrogen glove-box filled with nitrogen was used for taking aliquots of the content out of the tubes containing Sulfo-BSOCOES.

(2) OPH in pH 7.5, 0.1 M HEPES/50 μM $CoCl_2$, purified on 05/04/01, aliquoted in 1 ml, stored at –80°C, was thawed in a refrigerator. The enzyme stock contained 9223.30 units (0.29 mg OPH protein amount) per ml.

(3) An aliquot of 2 - 4 mg of 300 Å 2% NH_2-SAMMS in 1.8 ml tube was added with 100 – 200 μl of 3.2 mg/ml Sulfo-BSOCOES in pH 7.5, 0.1 M HEPES/50 μM $CoCl_2$, shaking for 2 min. Then, it was centrifuged at 1400 RPM for 2 min. and the supernatant containing the excess the cross linker was separated, the deposit was washed with 100 μl of the same buffer. Then it was added with 100 - 800 μl of the OPH stock, shaking at speed 1400 min^{-1} on Eppendorf Thermomixer 5436 at 25°C for 45 - 90 min.

(4) Then the enzyme incubation solution was separated and the resulting deposit was washed by n x 400 μl pH 7.5, 20 mM Phosphate/0.15 M NaCl ($n \geq 10$). In between, it was centrifuged at 14K RPM for 6 min. Finally, the washed deposit was resuspended in the same washing buffer again by 100 μl of the buffer per mg of original SAMMS. Both the suspension and the clear solution after the exhaustive washing were measured, indicating that the non-firmly immobilized OPH was completely washed out.

(5) The estimated protein amount of OPH covalently linked was 11.00 mg/g SAMMS by Pierce BCA Assay Kit (Pierce, 23227) with the initial specific activity 11386.07 Units per mg linked OPH.

Example 15

(1) 10.17 mg GOD (Sigma G-7016) was dissolved in 5 ml of pH7.5, 20 mM Phosphate/0.15 M NaCl as the GOD stock. The enzyme stock contained 395.05 units (1.05 mg OPH protein amount) per ml.

(2) Similar to Example 14, but used the GOD stock and 300 Å 20% NH_2-SAMMS instead.

(3) GOD activity was measured using Sigma standard method (revised on 08/30/96).

(4) The estimated protein amount of GOD covalently linked was 14.85 mg/g SAMMS, with the initial specific activity 114.31 Units per mg linked GOD.

Example 16

(1) 9.95 mg GOD (Sigma G-7016) was dissolved in 5 ml of pH 7.0, 20 mM Phosphate/0.15 M NaCl as the GOD stock. The enzyme stock contained 372.25 units (1.08 mg OPH protein amount) per ml.

(2) Similar to Example 15, but used 200 – 400 µl of 5% Glutaric Dialdehyde (Aldrich, 34085-5) in pH 7.0, 20 mM Phosphate/0.15 M NaCl instead of the sulfo-BSOCOES solution as the covalent linker source.

(3) The washing solution was pH 7.0 instead of pH 7.5 used in Example 15.

(4) The estimated protein amount of GOD covalently linked was 81.64 mg/g SAMMS, with the initial specific activity 72.34 Units per mg linked GOD.

Example 17

(1) GI, purified from Streptomyces rubiginosus, was obtained from Hampton Research, Inc. It was dialyzed in pH7.5, 20 mM Phosphate/0.15 M NaCl/1 mM $MgSO_4$, aliquoted in 1 ml, stored at –80°C and thawed in a refrigerator before use. The GI stock contained 180.22 units (4.11 mg GI protein amount) per ml.

(2) Similar to Example 15, but used the GI stock instead of the GOD stock. And the incubation was carried out at 40°C.

(3) The washing solution was pH7.5, 20 mM Phosphate/0.15 M NaCl/1 - 20 mM MgSO4.

(4) GI Activity definition at the present condition: 1 unit is 1 micromole D-Fructose converted to D-Glucose in one minute at pH 7.5, at 60°C, at 1.923 M D-Fructose concentration. The activity was measured in a general procedure as the following: 5-20 µl of the GI-SAMMS suspension was added with 0.5 ml 2M of D-Fructose (Fluka 47739) in pH 7.5, 20 mM Phosphate/0.15 M NaCl/20 mM $MgSO_4$, then shaking at speed 1400 min^{-1}, on Eppendorf Thermomixer 5436 at controlled temperature 60°C for 15 min. Then, stopped the reaction by 10 µl of 1.8 M H_2SO_4. Next, the reacted solution 5 - 20 µl was added 2.0 ml Glucose (GO) Assay Reagent (Sigma, G3660) and allowed to incubate in water bath at 37°C for 30 min. The incubation reaction was stopped by 1.0 ml of 6 M H_2SO_4 and the absorbance was

measured at 540 nm. From the glucose standard curve, obtained the glucose amount produced from the GI catalyzed conversion of Fructose.

(5) The estimated protein amount of GI covalently linked was 48.47 mg/g SAMMS, with the initial specific activity 53.40 Units per mg linked GOD.

Example 18

(1) Similar to Example 17, but used 200 – 400 µl of 5% Glutaric Dialdehyde (Aldrich, 34085-5) in pH 7.5, 20 mM Phosphate/0.15 M NaCl instead of the sulfo-BSOCOES solution as the covalent linker source.

(2) The estimated protein amount of GI covalently linked was 30.45 mg/g SAMMS, with the initial specific activity 39.19 Units per mg linked GOD.

Example 19

(1) purified OPH in pH 7.5, 0.1 M HEPES/50 µM $CoCl_2$, aliquoted in 1 ml, stored at – 80°C, was thawed in a refrigerator. The enzyme stock contained 9223.30 units (0.29 mg OPH protein amount) per ml.

(2) An aliquot of 2 - 4 mg of 300 Å 2% NH_2-SAMMS in 1.8 ml tube was added with 100 - 800 µl of the OPH stock, shaking at speed 1400 min^{-1} on Eppendorf Thermomixer 5436 at 25°C for 2 – 3 h.

(3) Then the enzyme incubation solution was separated and the resulting deposit was washed by n x 400 µl pH 7.5, 20 mM Phosphate/0.15 M NaCl ($n \geq 10$). In between, it was centrifuged at 14,000 RPM for 6 min. Finally, the washed deposit was resuspended in the same washing buffer again by 100 µl of the buffer per mg of original SAMMS. Both the suspension and the clear solution after the exhaustive washing were measured, indicating that the non-firmly immobilized OPH was completely washed out.

(4) The estimated protein amount of OPH entrapped was 9.52 mg/g SAMMS by Pierce BCA Assay Kit, with the initial specific activity 16378.74 Units per mg entrapped OPH.

Example 20

(1) 10.17 mg GOD (Sigma G-7016) was dissolved in 5 ml of pH7.5, 20 mM Phosphate/0.15 M NaCl as the GOD stock. The enzyme stock contained 395.05 units (1.05 mg OPH protein amount) per ml.

(2) Similar to Example 19, but used the GOD stock and 300 Å 20% NH_2-SAMMS instead.

(3) GOD activity was measured using Sigma standard method (revised on 08/30/96).

(4) The estimated protein amount of GOD entrapped was 13.35 mg/g SAMMS, with the initial specific activity 99.33 Units per mg entrapped GOD.

Example 21

(1) Similar to Example 20. But 9.95 mg GOD (Sigma G-7016) was dissolved in 5 ml of pH 7.0, 20 mM Phosphate/0.15 M NaCl as the GOD stock. The enzyme stock contained 372.25 units (1.08 mg OPH protein amount) per ml.

(2) The washing solution was pH 7.0 instead of pH 7.5 used in Protocol 7.

(3) The estimated protein amount of GOD entrapped was 14.59 mg/g SAMMS, with the initial specific activity 136.28 Units per mg entrapped GOD.

Example 22

(1) GI, purified from Streptomyces rubiginosus, was obtained from Hampton Research, Inc. It was dialyzed in pH7.5, 20 mM Phosphate/0.15 M NaCl/1 mM MgSO4, aliquoted in 1 ml, stored at –80°C and thawed in a refrigerator before use. The GI stock contained 180.22 units (4.11 mg GI protein amount) per ml.

(2) Similar to Example 20, but used the GI stock instead of the GOD stock. And the incubation was carried out at 40°C.

(3) The washing solution was pH7.5, 20 mM Phosphate/0.15 M NaCl/1 - 20 mM MgSO4.

(4) GI Activity was measured in the same way as did in Protocol 4.

(5) The estimated protein amount of GI entrapped was 94.94 mg/g SAMMS, with the initial specific activity 43.26 Units per mg entrapped GOD.

Example 23

(1) Purified OPH, aliquoted in 2 ml, stored at –80°C, was thawed, dialyzed from pH 8.5, 25 mM HEPES/20 % glycerol/50 μM $CoCl_2$ to pH 7.2, 0.1 M HEPES. The enzyme stock contained 4830.00 units (0.39 mg OPH protein amount) per ml.

(2) An aliquot of 82.45 mg of 300 Å 2% HOOC-SAMMS in 50 ml tube was added with 12 ml of the OPH stock, shaking at speed 350 RPM on Innova 4330 refrigerated incubator shaker at 25°C for 2 h 47 min. Then, the resulting suspension was aliquoted in 0.4 ml.

(3) Then the enzyme incubation solution was separated and the resulting deposit was washed by n x 400 μl pH 7.5, 20 mM HEPES ($n \geq 10$). In between, it was centrifuged at 14,000 RPM for 6 min. Finally, the washed deposit was resuspended in the same washing buffer again by 100

μl of the buffer per mg of original SAMMS. Both the suspension and the clear solution after the exhaustive washing were measured, indicating that the non-firmly immobilized OPH was completely washed out.

(4) The estimated protein amount of OPH entrapped was 46.73 mg/g SAMMS, with the initial specific activity 26766.78 Units per mg entrapped OPH.

Example 24

(1) 31.07 mg GOD (Sigma G-7016) was dissolved in 11 ml of pH7.0, 20 mM Phosphate as the GOD stock. . The enzyme stock contained 393.08 units (1.71 mg GOD protein amount) per ml.

(2) Similar to Example 23, but used the GOD stock and 98.67 mg of 300 Å 20% NH_2-SAMMS instead.

(3) The washing solution was pH 7.0, 20 mM Phosphate buffer.

(4) GOD activity was measured using Sigma standard method (revised on 08/30/96).

(5) The estimated protein amount of GOD entrapped was 107.94 mg/g SAMMS, with the initial specific activity 76.22 Units per mg entrapped GOD.

Example 25

(1) GI, purified from Strptomyces rubiginosus, was obtained from Hampton Research, Inc. It was dialyzed in pH7.5, 20 mM Phosphate/0.15 M NaCl/1 mM MgSO4, aliquoted in 1 ml, stored at –80°C. Six aliquots were thawed in a refrigerator before use and diluted together by 22.5 ml of pH 7.0, 20 mM Sodium/1 mM $MgSO_4$ as the GI stock. The GI stock contained 42.10 units (0.86 mg GI protein amount) per ml.

(2) Similar to Example 24, but used the GI stock and 100.98 mg of 300 Å 20% NH_2-SAMMS. And the incubation was carried out at 40°C.

(3) The washing solution was pH7.5, 20 mM Phosphate/1 - 20 mM MgSO4.

(4) GI Activity was measured in the same way as did in Example 17.

(5) The estimated protein amount of GI entrapped was 77.26 mg/g SAMMS, with the initial specific activity 45.06 Units per mg entrapped GI.

Results of Testing Including Comparative Examples and Varying Conditions

Normal Silica (Item#: BMSI 1203), which is commercially available from PolyLC, Inc, was functionalized in a similar way for SAMMS and used to replace the SAMMS in the protocols mentioned above under the same experimental conditions for comparison.

For investigating pore size, functional types and coverage effects, different pore sizes, functional monolayer types and coverages were used to replace the SAMMS in the protocols mentioned above under the same experimental conditions.

A variety of different washing solutions were used to replace the washing solution in the protocols mentioned above under the same experimental conditions for investigating the effects of the buffer composition, pH, stability and ion strength.

Some results are shown in the figures. Note the following notations were used: 300 Å 20% NH_2-SAMMS means a pore size of 300 Å mesoporous silica with a 20% coverage of NH_2-functional monolayer derivatized from tris-(methoxy)aminopropylsilane, while 300 Å 2% HOOC-SAMMS means the same mesoporous silica with a 2% coverage of HOOC- functional monolayer from tris-(methoxy)carboxylethylsilane.

Fig. 5 shows comparative data for OPH immobilized on 5 different matrices. The three examples using normal silica had activities of 48 units per mg silica while the OPH immobilized on SAMMS demonstrated activities of 156 and 125. See Examples 14 and 19. Thus, the protein systems made from normal silica demonstrated significantly lower activities.

Type of functional group and degree of functionalization were found to be important factors in forming protein systems. When example 19 (activity 156) was repeated with 20 % functionalization, instead of 2%, (on either 100 Å, 170 Å or the 300 Å SAMMS), activities of less than 1 were obtained. Similarly, when Example 14 (activity 125) was repeated with 20 % functionalization, instead of 2%, (on either 100 Å, 170 Å or the 300 Å SAMMS), activities of less than 20 were obtained. A 300 Å 20% HOOC-SAMMS treated with OPH demonstrated an activity of 97 while a 300 Å 20% NH_2-SAMMS similarly treated with OPH had an activity less than 1.

The data in the Examples section demonstrates the importance of tailoring anionic or cationic functionalization at the appropriate density for individual proteins, independent of whether covalent immobilization or noncovalent entrapment of the protein is utilized (although the type of bonding is another factor that can be controlled). This tailorability facilitates incorporating high concentrations of active protein into a porous matrix. The resulting protein system can be defined in terms of its activity or other measurable properties.

Figs. 6 and 9 show that activity is sensitive to buffer concentration (ionic strength) both for proteins entrapped at varying buffer concentrations (Fig. 6) and specific activity, relative to a solution of OPH at pH 7.2, of entrapped proteins at varying buffer concentrations (Fig. 9).

As shown in Figs. 7 and 8, a sample prepared at pH 7.0 contained more OPH than samples prepared at higher pH, while the specific activity of the protein system appeared to be optimized near pH 7.5. The specific activity in Fig. 8 is the specific activity of the immobilized OPH divided by the specific activity of a reference solution of OPH at pH 7.2.

Whether held by non-covalent bonds or by covalent bonding, entrapped OPH demonstrated significantly better stability as compared to OPH in solution. As shown in Fig. 10, entrapped protein showed a loss of less than about 30% activity when refrigerated at 4 °C for 110 days. Some activity was recovered, presumably through renaturing, after 40 additional days at room temperature.

Fig. 11 shows comparative data for GOD immobilized on 6 different matrices. The three examples using normal silica had activities of 0.2, 0.3 and 0.4 while the GOD immobilized on SAMMS demonstrated activities of 2.0 and 5.9. See Examples 20 and 15. Thus, the protein systems made from normal silica demonstrated significantly lower activities. The unfunctionalized SAMMS, labeled "300 Å Meso-Silica," (i.e., 0% NH_2) treated under the same conditions showed an activity of only 0.3. A GOD protein system prepared from 100 Å 20% NH_2-SAMMS showed the same activity as 300 Å 20% NH_2-SAMMS, but a GOD protein system prepared from 100 Å 20% NH_2-SAMMS with a covalent cross-linker showed an activity of 4.0 as compared to the 5.9 activity of the analogously-prepared protein covalently cross-linked in 300 Å 20% NH_2-SAMMS. As shown in Fig. 12, for GOD, a porous matrix with a 20% amino surface coverage performed significantly better than a 2% surface coverage. The presence of 0.15 M sodium chloride caused a substantial decrease in activity of GOD in the NH_2-SAMMS system. As shown in Fig. 13, increasing pH during preparation of the GOD-NH_2-SAMMS system resulted in decreased activity; however, the specific activity of GOD was not reduced at the higher pH range (see Fig. 14). Again, as shown in Fig. 15, the protein system showed enhanced stability relative to the GOD solution, and, surprisingly, the non-covalently bonded protein system exhibited better stability as compared with the covalently-bonded systems after refrigeration at 4 °C.

Thus, alternatively or in addition to functionalization, it may be desirable to characterize each protein system in terms of covalent and noncovalent bonding. In some preferred embodiments, the non-covalently bonded protein has superior stability relative to the covalently cross-linked system when tested according to the procedures described herein.

Fig. 16 shows comparative data for GI immobilized on 8 different matrices. The four examples prepared under analogous conditions using normal silica had activities of 0.0, 0.0, 0.3 and 0.0 while the GI immobilized on SAMMS demonstrated activities of 4.1, 1.2 and 2.6, respectively. See Examples 22, 17 and 18. Thus, again, the protein systems made from normal silica demonstrated significantly lower activities. The unfunctionalized SAMMS, labeled "300 Å Meso-Silica," (i.e., 0% NH_2) treated under the same conditions showed an activity of 0.0. For GI, a significantly larger effect was seen based on pore size (Fig. 17), which was not unexpected in view of GI's larger size. As shown in Fig. 18, GI immobilized on covalently-linked 20% NH_2-normal silica demsonstrated a specific activity of 70, while a similar preparation of GI

immobilized on covalently-linked 300 Å 20% NH_2-SAMMS demonstrated a specific activity of 89. GI in NH_2-SAMMS was found to be nearly unaffected by the presence of 0.15 M sodium chloride. 300 Å 2% NH_2-SAMMS and 300 Å 20% HOOC-SAMMS were found to have little or no activity.

GI is a robust enzyme and entrapment in SAMMS showed relatively little stability enhancement for the time period and conditions tested. Based on stability testing in OPH protein systems, however, it is reasonable to conclude that testing under more challenging conditions, such as exposure to denaturants, would reveal the superior stability properties of preferred inventive protein systems, especially SAMMS-based systems. The invention can be generally described relative to stability under various testing conditions.

This invention may include various modifications and alterations without departing from the spirit and scope of the invention. Thus, it should be understood that the invention is not to be limited to the specific descriptions and examples, but it is to be controlled by the limitations set forth in the following claims and equivalents of the elements set forth in the claims.

CLAIMS

We claim:

1. A protein system comprising:

a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, and

further comprising a chemically-active protein bonded to the matrix material.

2. The system of claim 1 wherein the protein system comprises 0.01 to 1 mmol of said protein per gram of matrix material and wherein said protein in the protein system exhibits an activity of at least 65% that of the activity of the protein in the active state.

3. The system of claim 1 wherein said protein occupies between 5 and 40% of the average pore volume.

4. The system of claim 3 wherein the protein system comprises 0.01 to 1 mmol of said protein per gram of matrix material and wherein at least 90% of the pore volume is composed of pores having sizes in the range of 100 to 200 Å.

5. The system of claim 3 wherein said protein is an enzyme.

6. The system of claim 5 wherein the enzyme has a volume in the range of 0.5×10^5 Å^3 to 3×10^5 Å^3.

7. The system of claim 6 wherein said enzymes have activities of at least 50% that of the active state.

8. The system of claim 7 wherein said enzyme is OPH having an activity of 60 to 95% that of the active state.

9. The system of claim 6 wherein the volume of the protein is in the range of 10 to 25% of the average pore volume.

10. The system of claim 6 wherein the surface area of the porous matrix material is at least 700 m^2/g.

11. The system of claim 5 wherein the enzyme is OPH, and wherein said system comprises between 5 and 25 mg OPH per cubic centimeter.

12. The system of claim 5 wherein the enzyme is OPH, and having a V_{max} of 0.15 to 0.66.

13. The system of claim 5 wherein the system retains about 10% of its activity after 24 hours under alkaline conditions.

14. The system of claim 2 wherein the matrix is a SAMMS.

15. A chemical process catalyzed by the system of claim 1.

16. A protein system comprising:

a porous matrix material being sized such that the protein system comprises 0.01 to 1 mmol of protein per gram of matrix material and wherein said protein in the protein system exhibits an activity of at least 65% that of the activity of the protein in the active state.

17. The protein system of claim 16 wherein the porous matrix material has a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å.

18. The protein system of claim 17 wherein said protein occupies between 5 and 40% of the average pore volume.

19. The protein system of claim 17 wherein said enzyme is OPH.

20. The protein system of claim 17 wherein the matrix is a mesoporous oxide material.

21. A chemical process catalyzed by the system of claim 16.

22. A method of forming a protein system comprising the steps of:

providing a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, and

reacting the porous matrix material with a protein so that said protein chemically bonds to the porous matrix material.

23. The method of claim 22 wherein the porous matrix material comprises surface hydroxyls and further comprising the step of reacting said surface hydroxyls with a coupling agent to form a functionalized monolayer.

24. The method of claim 23 wherein said functionalized monolayer comprises reactive moieties selected from the group consisting of mercapto, amino, carboxyl, hydroxyl, and azido.

25. The method of claim 23 wherein the coupling agent comprises mercaptopropyltrimethoxysilane.

26. The method of claim 23 wherein the coupling agent has a chain length of 2 to 20 atoms.

27. The method of claim 22 wherein the porous matrix material, prior to the step of reacting, has a surface area of at least 900 m^2/g.

28. A protein system made by the method of claim 22.

29. A method for producing OPH comprising:

transfecting a host cell with said vector comprising a sequence encoding OPH, said sequence being operably linked to a T7 expression control sequence,

culturing said transfected host cell under conditions permitting expression under the control of said expression control sequence, and

purifying said OPH from the cell or the medium of the cell.

30. The method of claim 29 wherein the vector is provided with the sequence encoding OPH operably linked to the T7 expression control sequence.

31. The method of claim 29 wherein the OPH has an activity of about 13,000 units/mg.

32. The method of claim 29 wherein said vector is a plasmid.

33. The method of claim 30 wherein the vector is a plasmid.

34. The method of claim 29 wherein said host cell is a prokaryotic cell.

35. The method of claim 34 wherein said prokaryotic cell is a bacterium.

36. The method of claim 35 wherein said bacteria is *Escherichia coli*.

37. The method of claim 29 wherein said host cell is a eukaryotic cell.

38. The method of claim 37 wherein said eukaryotic cell is a yeast cell.

39. The method of claim 38 wherein said yeast cell is *Pichia pastoris*.

40. The method of claim 22 comprising:

reacting the porous matrix material with a cross-linking agent to form a porous matrix material having cross-linking agents covalently bound to the surface, and

reacting the porous matrix material having cross-linking agents covalently bound to the surface with a protein so that said protein chemically bonds to the porous matrix material.

41. A method of forming a protein system comprising the steps of:

providing a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å,

wherein the porous matrix material has a functionalized surface, and

adding a protein so that said protein is entrapped by non-covalent bonding in the porous matrix material.

42. The method of claim 41 wherein the porous matrix material comprises a functionalized monolayer that comprises reactive moieties selected from the group consisting of mercapto, amino, carboxyl, hydroxyl, and azido.

43. The method of claim 41 wherein the protein is added under conditions such that the protein in the resulting protein system has an activity that is at least 50% of the protein in the active state.

44. A protein system comprising:

a porous matrix material and comprising a protein disposed within the porous matrix material;

wherein the protein system comprises at least 0.01 mmol of protein disposed within the porous matrix material per gram of matrix material and wherein said protein system exhibits an

activity at least 2 times greater than the activity of a protein system that has been formed under identical conditions on a normal silica matrix material.

45. The protein system of claim 44 wherein the porous matrix material is disposed in a microchannel.

46. Apparatus comprising the protein system of claim 45.

47. The protein system of claim 44 wherein said protein system exhibits an activity at least 10 times greater than the activity of a protein system that has been formed under identical conditions on a normal silica matrix material.

48. The protein system of claim 44 wherein the porous matrix material being sized such that the protein system comprises 0.01 to 1 mmol of protein per gram of matrix material and wherein said protein in the protein system exhibits an activity of at least 65% that of the activity of the protein in the active state.

49. The protein system of claim 44 wherein the protein loses less than about 30% activity when refrigerated at 4 °C for 110 days.

50. The protein system of claim 44 wherein the activity, measured per protein molecule, is at least 60% of the protein's activity on the active state.

51. The protein system of claim 44 wherein the protein is non-covalently bonded in the porous matrix.

52. The protein system of claim 44 wherein the protein has a molecular weight in the range of 8,000 to 300,000 daltons.

53. The protein system of claim 44 wherein the protein has a molecular weight of at least 8,000 daltons.

54. The protein system of claim 44 wherein the porous matrix material is functionalized with a surface coverage of 2 to 80%.

55. The protein system of claim 44 made by providing a porous matrix material having a pore volume wherein at least 90% of the pore volume is composed of pores having sizes in the range of 50 to 400 Å, wherein the porous matrix material has a functionalized surface, and adding a protein so that said protein is entrapped by non-covalent bonding in the porous matrix material.

56. The protein system of claim 44 wherein the protein is entrapped by covalent bonding through a cross-linking agent to the porous matrix material.

57. The protein system of claim 44 wherein the porous matrix material comprises a SAMMS.

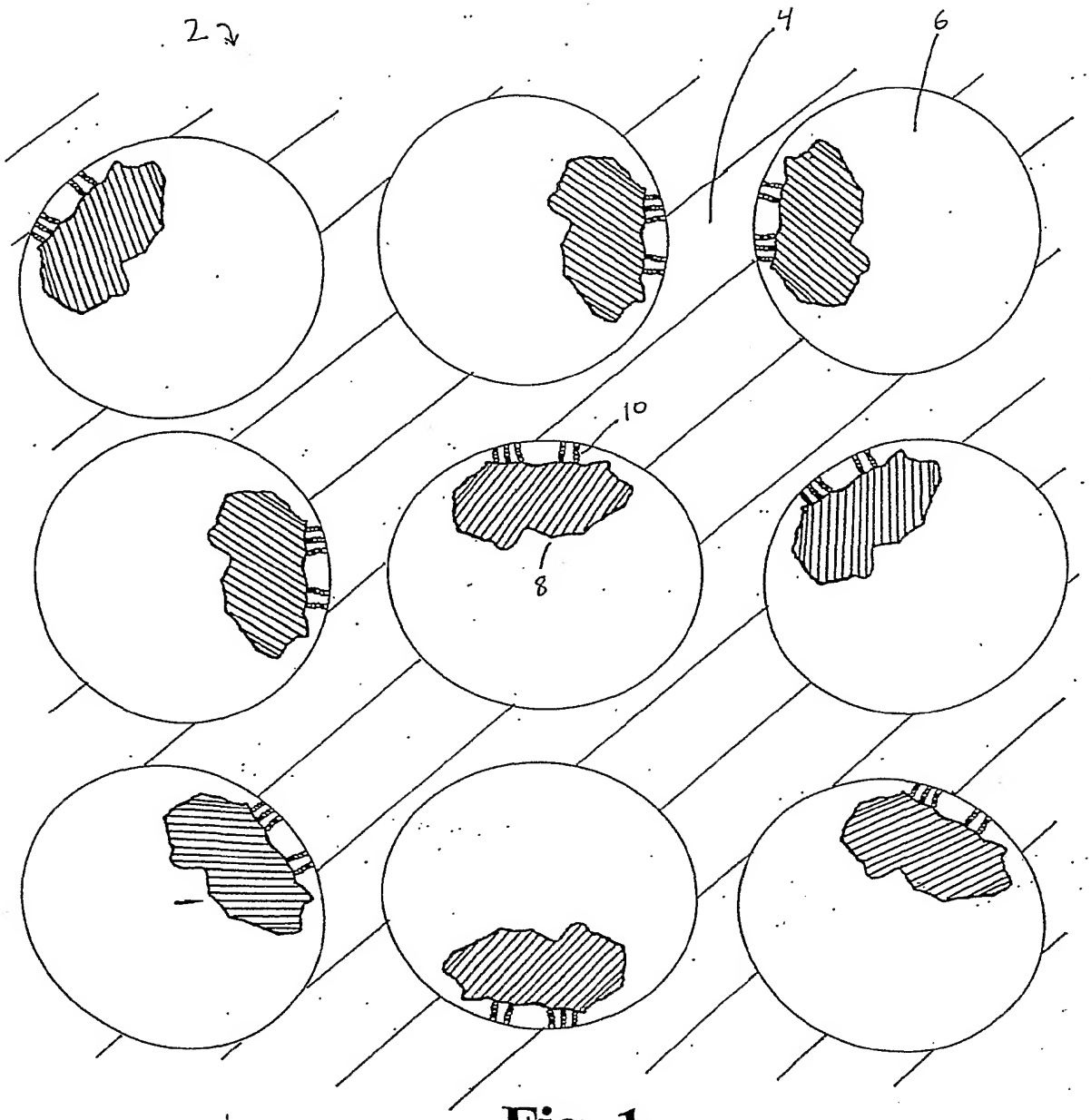

Fig. 1

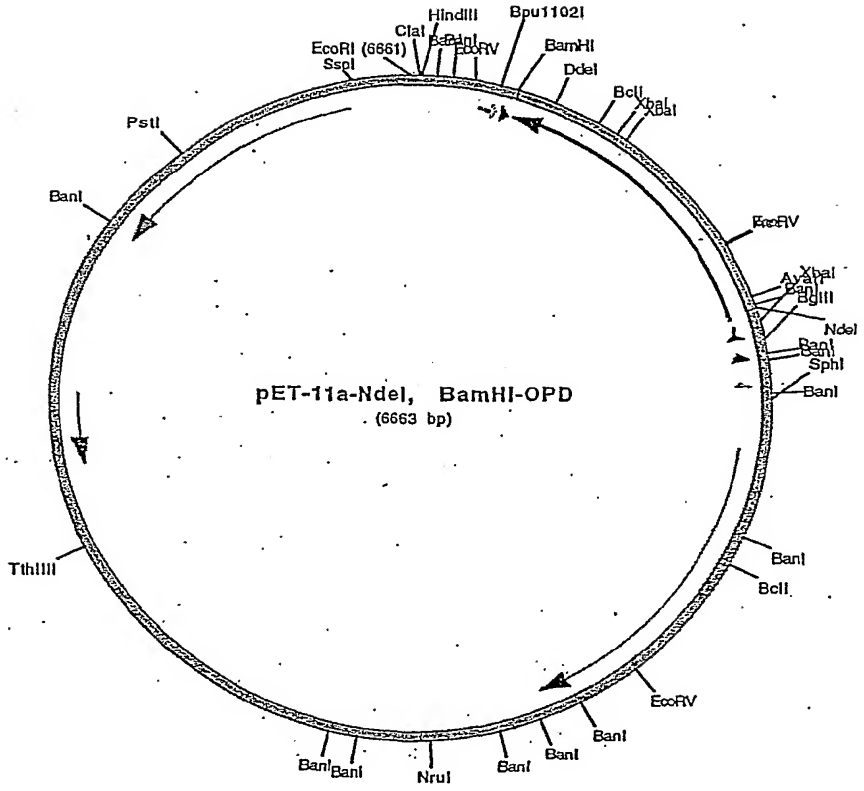
pET-11a-NdeI, BamHI-OPD
(6663 bp)
HindIII
Bpu1102I
ClaI
EcoRI (6661)
SspI
BamHI
DdeI
BclI
XbaI
PstI
BanI
EcoRV
XbaI
AvaII
BanI
BglII
NdeI
BanI
SphI
BanI
BanI
BclI
EcoRV
BanI
BanI
BanI
NruI
BanI
BanI
TthIII

Fig. 2

CGTCATGACGCCCGCAAGGTCGGTGACAAGAACCGCGCCGGGTTAGTCACAGTGATGCCTGCCAGCGTTTGCTGTGGGAC
GCCCTTCTCTCGTAGGAATGGGATCACTCTCAGTGGAATGAAGGCCATCCCGTCGGGGTTCACGCGATCCATCACGTCCA
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GCCGCCACGATATGAACGTCGGCAGCCCGCGAAACCTCGGCCAATAAACTGACGTCGCGACCGATATCGAAAGTCGACAC
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CGAAGAACTCTGGCCAAGCACGCAAGAATCCTGCCGAGCTGCCGCAGATGTGCTCGTGAGTCAGTGTGAAACCCGCTTCA
GAGATTGTGATAGGACCACGAACGGTGTTGATACGGTCACCGGTACCGATAGACATATGTATATCTCCT

Fig. 3

MSIGTGDRINTVRGPITISEAGFTLTHEHICGSSAGFLRAWPEFFGSRKALAEKAVRGLRRARAAGVRTIVDVSTFDIGR
DVSLLAEVSRAADVHIVAATGLWFDPPLSMRLRSVEELTQFFLREIQYGIEDTGIRAGIIKVATTGKATPFQELVLKAAA
RASLATGVPVTTHTAASQRDGEQQAAIFESEGLSPSRVCIGHSDDTDDLSYLTALAARGYLIGLDHIPHSAIGLEDNASA
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Fig. 4

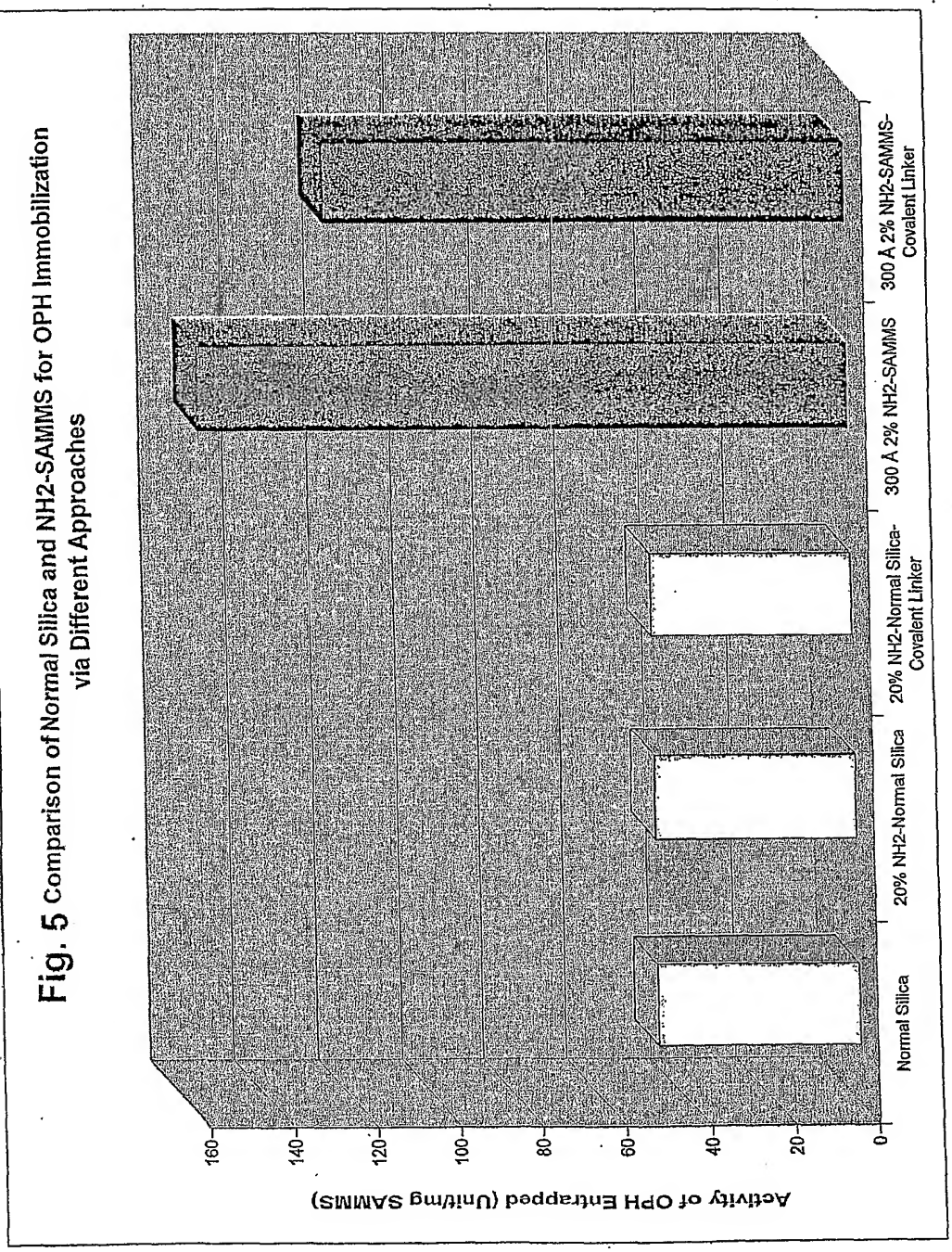

Fig. 5 Comparison of Normal Silica and NH2-SAMMS for OPH Immobilization via Different Approaches

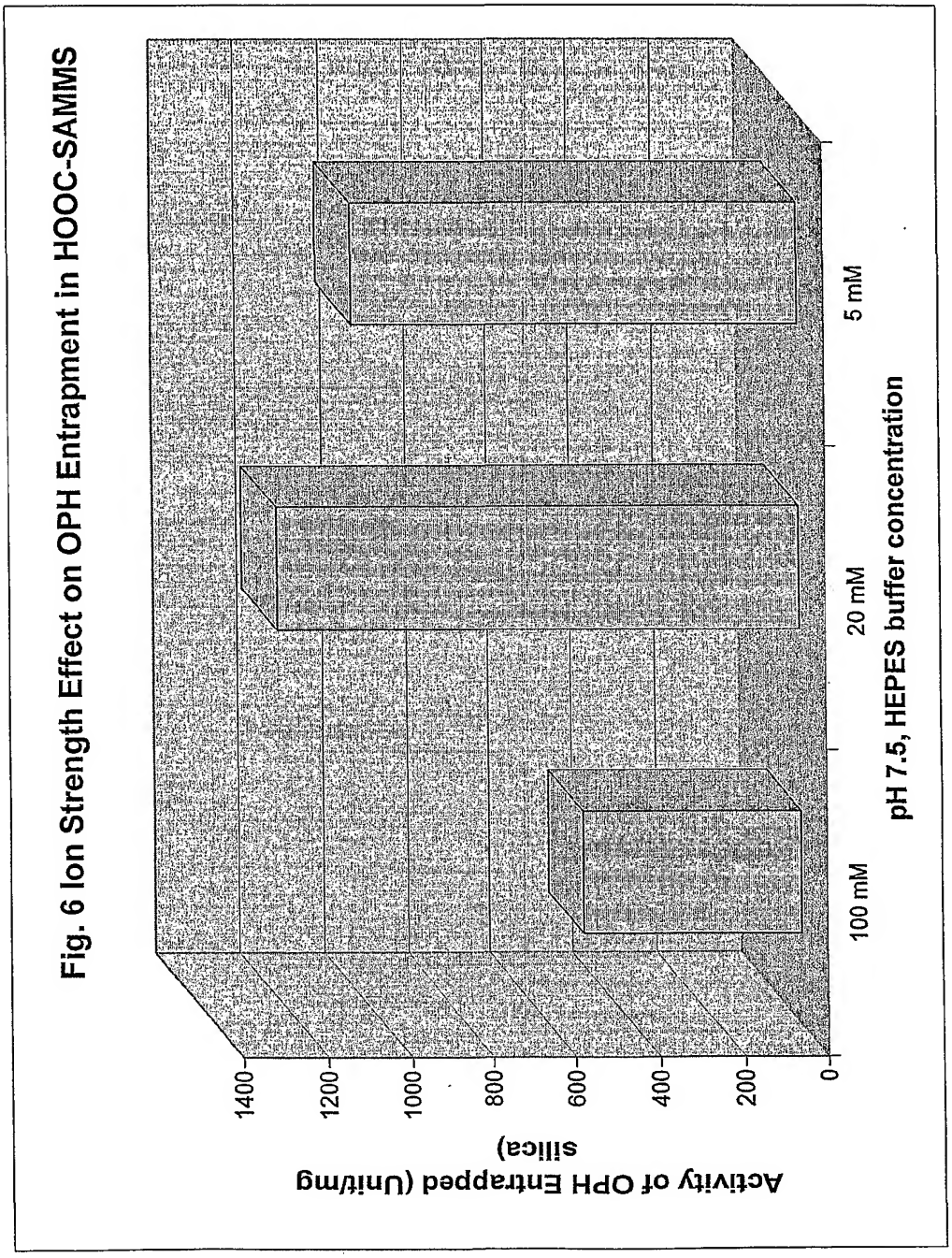
Fig. 6 Ion Strength Effect on OPH Entrapment in HOOC-SAMMS
Activity of OPH Entrapped (Unit/mg silica)
1400
1200
1000
800
600
400
200
0
100 mM
20 mM
5 mM
pH 7.5, HEPES buffer concentration

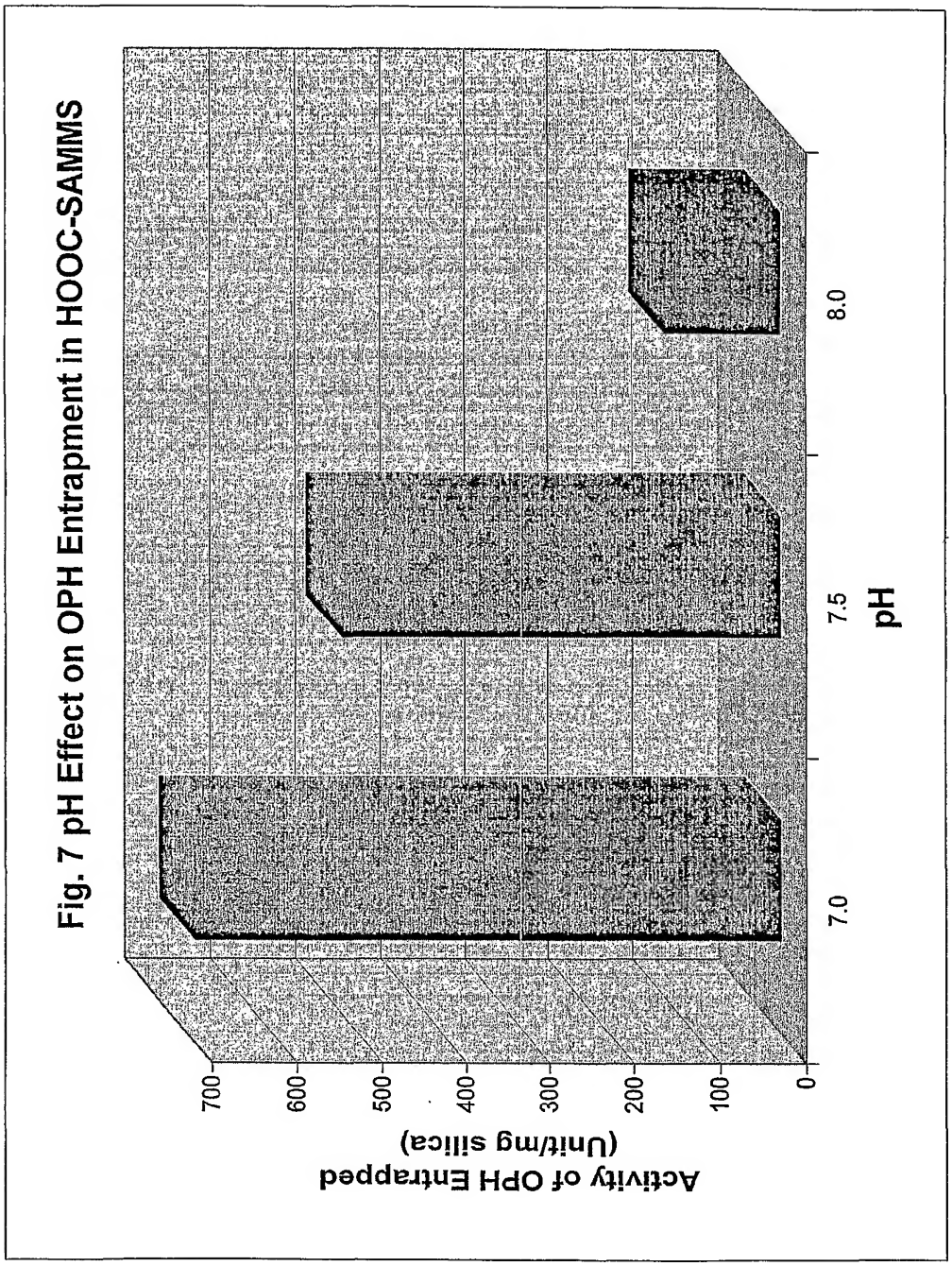
Fig. 7 pH Effect on OPH Entrapment in HOOC-SAMMS
Activity of OPH Entrapped
(Unit/mg silica)
0
100
200
300
400
500
600
700
7.0
7.5
8.0
pH

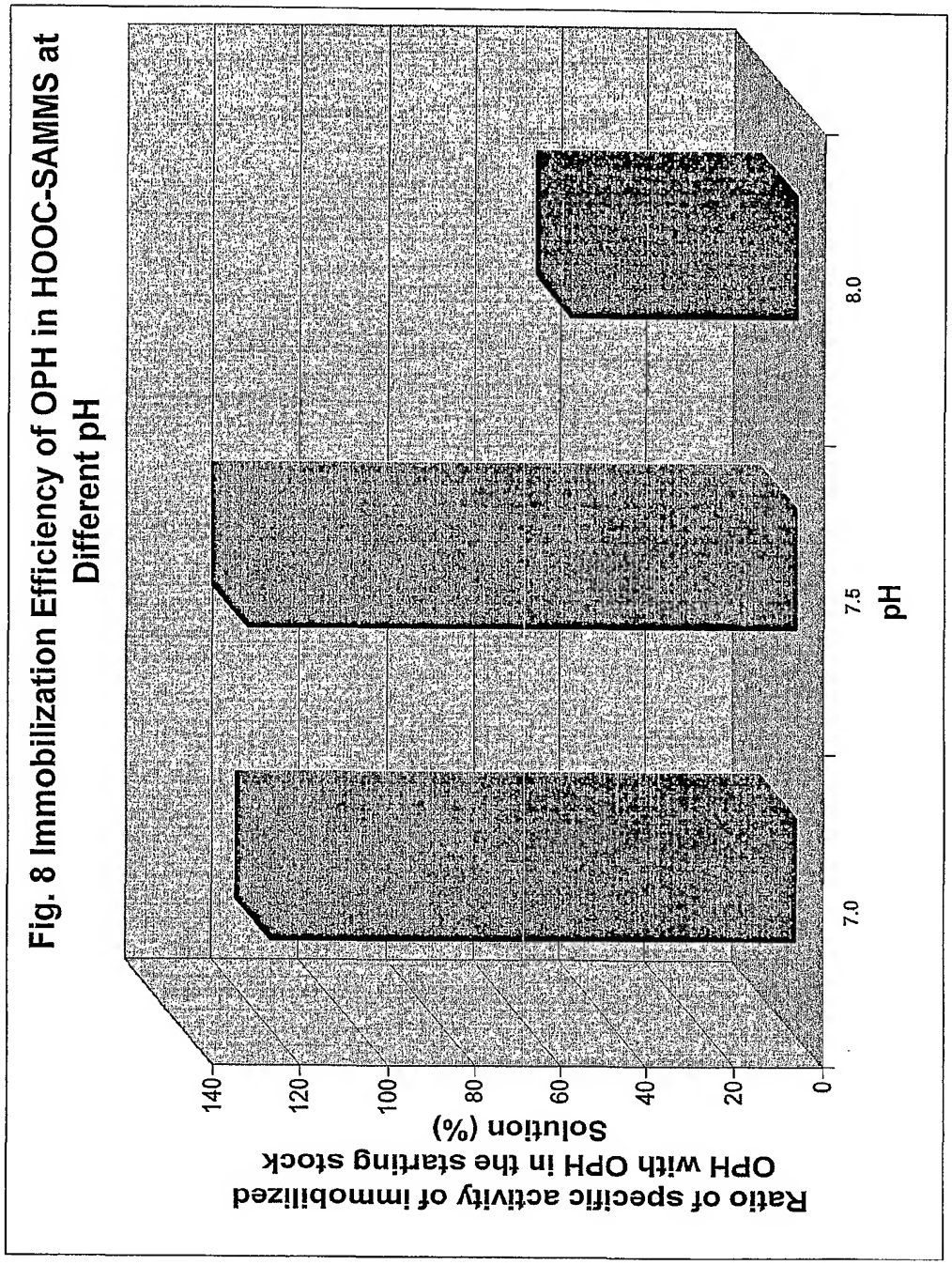
Fig. 8 Immobilization Efficiency of OPH in HOOC-SAMMS at Different pH
Ratio of specific activity of immobilized OPH with OPH in the starting stock Solution (%)
140
120
100
80
60
40
20
0
7.0
7.5
8.0
pH

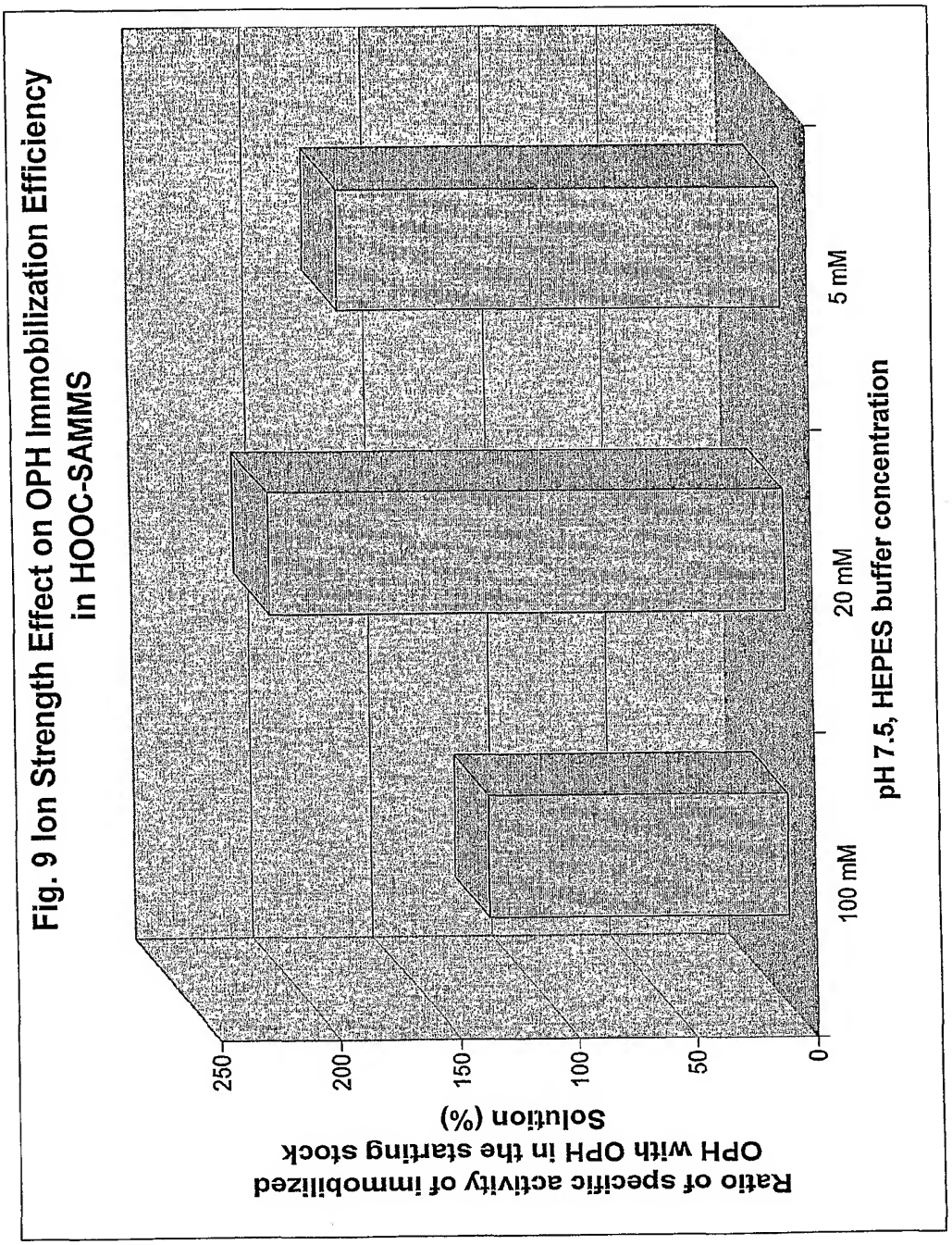
Fig. 9 Ion Strength Effect on OPH Immobilization Efficiency in HOOC-SAMMS
Ratio of specific activity of immobilized OPH with OPH in the starting stock Solution (%)
250
200
150
100
50
0
100 mM
20 mM
5 mM
pH 7.5, HEPES buffer concentration

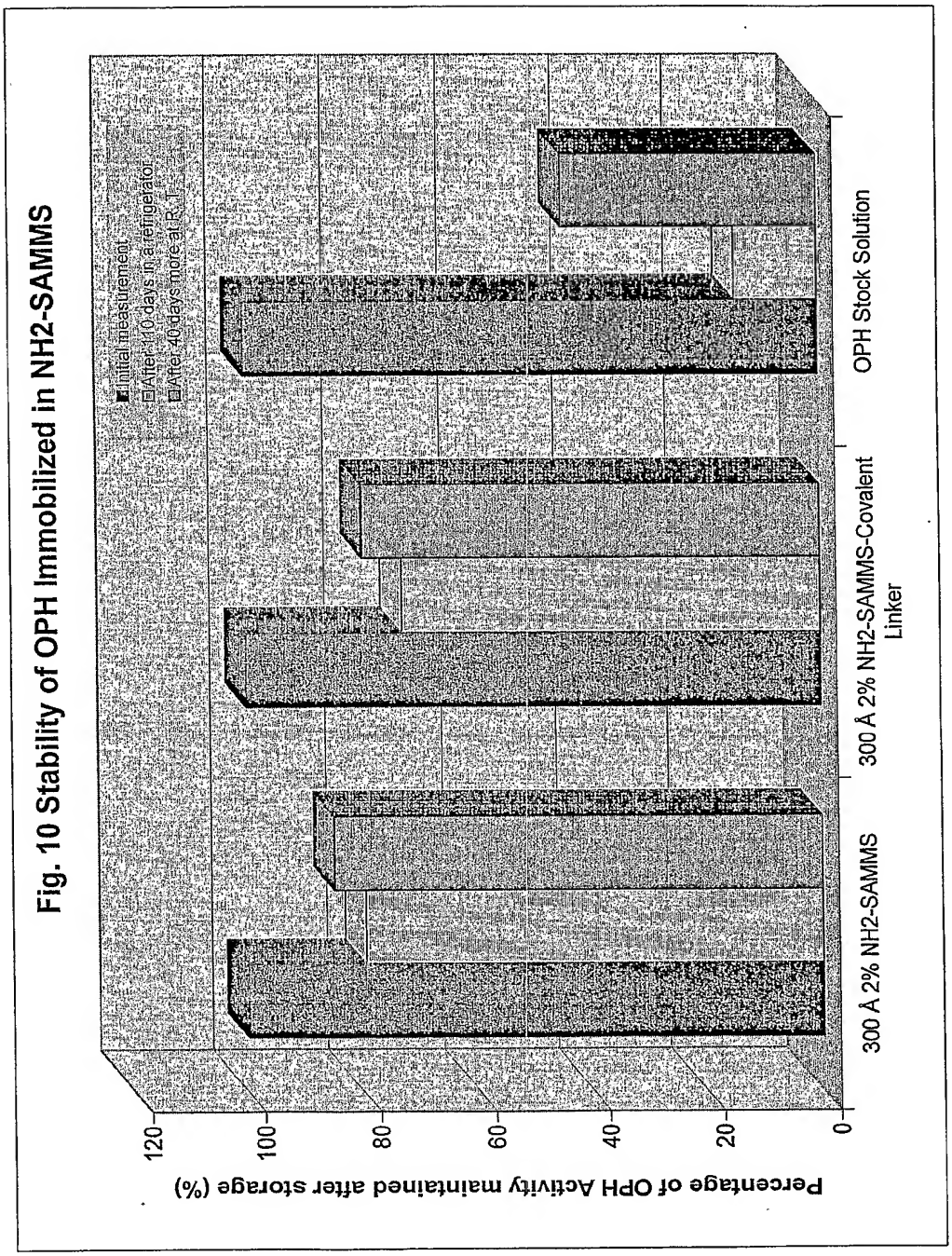
Fig. 10 Stability of OPH Immobilized in NH2-SAMMS
Initial measurement
After 10 days in a refrigerator
After 40 days more at R.T.
Percentage of OPH Activity maintained after storage (%)
0
20
40
60
80
100
120
300 Å 2% NH2-SAMMS
300 Å 2% NH2-SAMMS-Covalent Linker
OPH Stock Solution

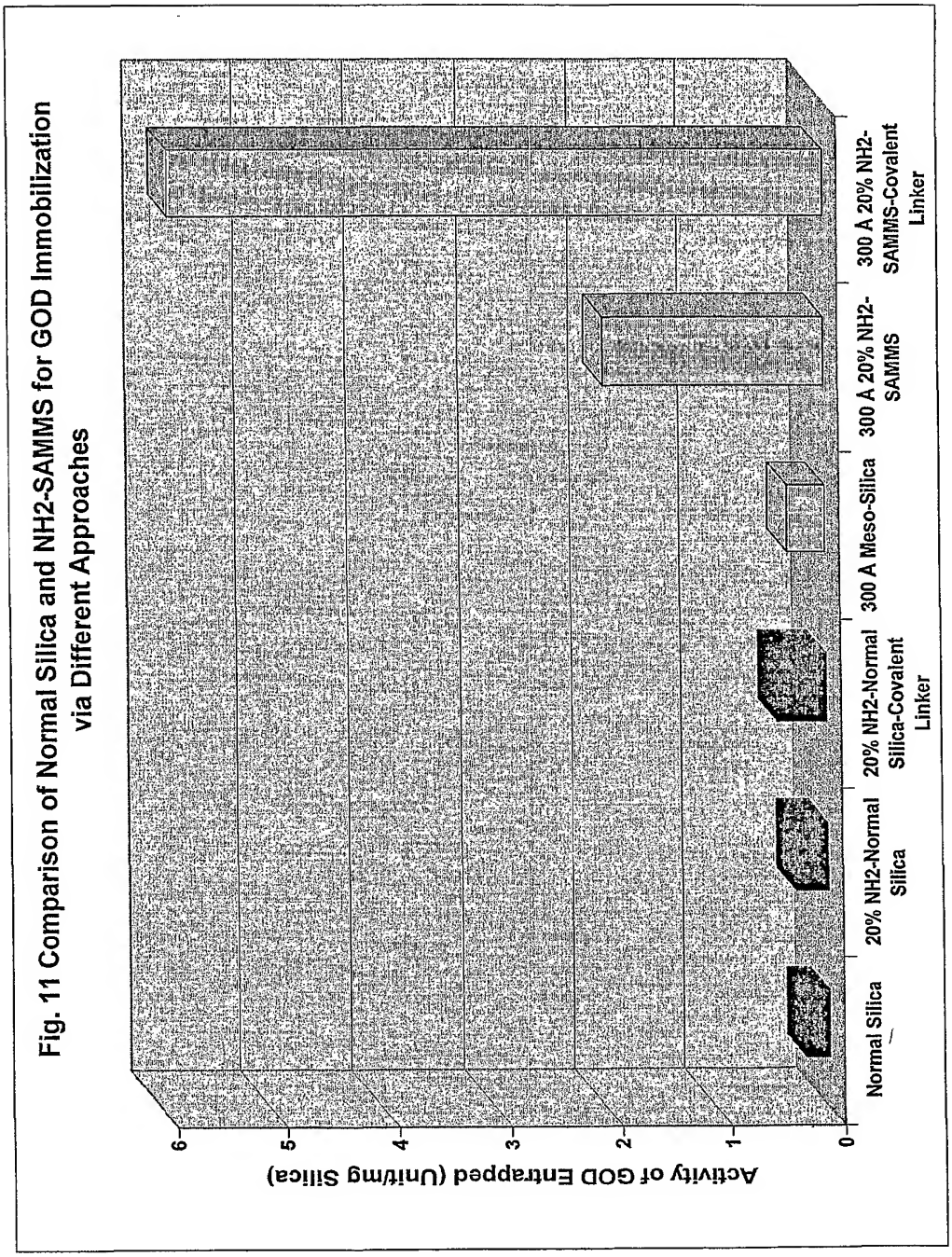

Fig. 11 Comparison of Normal Silica and NH2-SAMMS for GOD Immobilization via Different Approaches

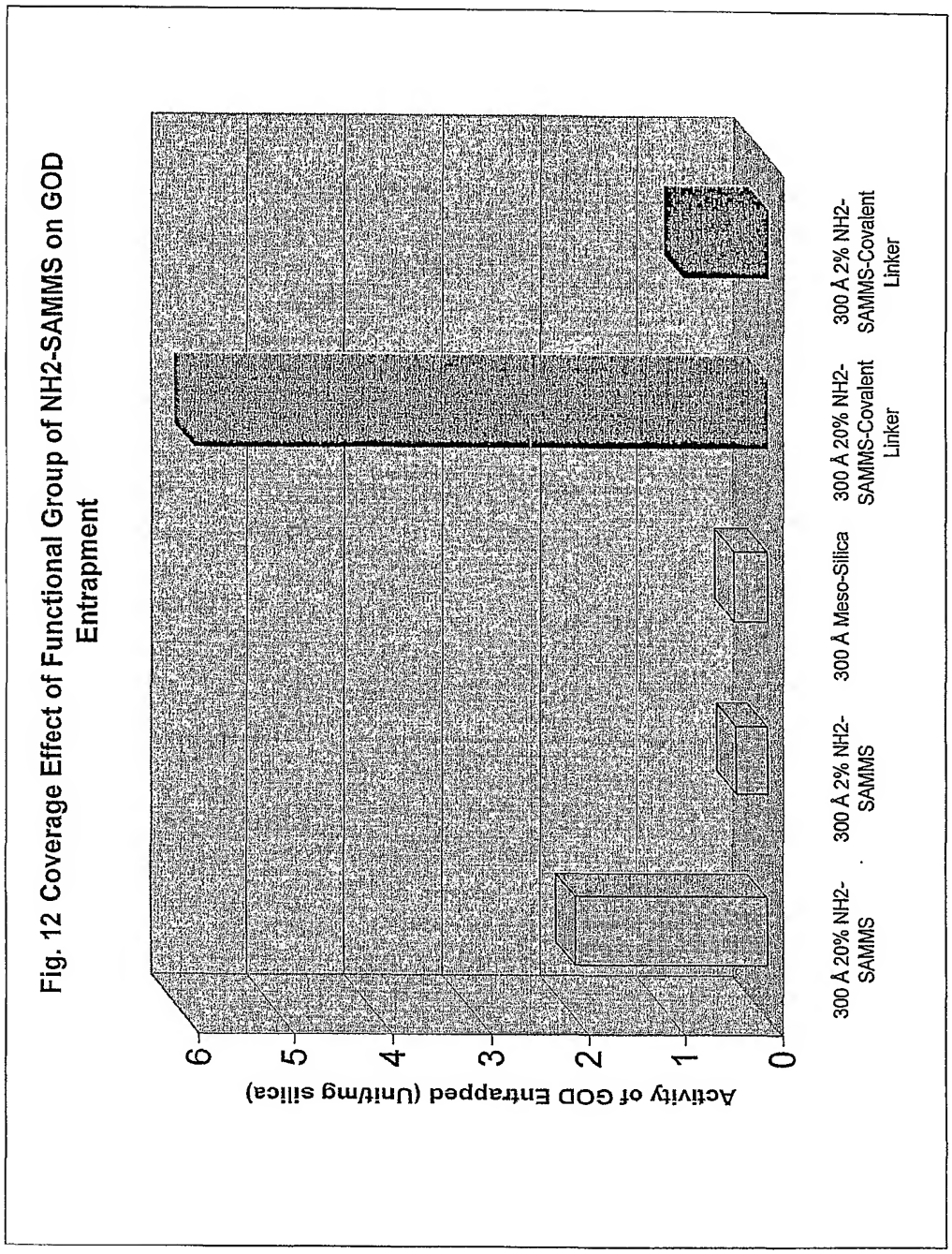
Fig. 12 Coverage Effect of Functional Group of NH2-SAMMS on GOD Entrapment
Activity of GOD Entrapped (Unit/mg silica)
0
1
2
3
4
5
6
300 Å 20% NH2-SAMMS
300 Å 2% NH2-SAMMS
300 Å Meso-Silica
300 Å 20% NH2-SAMMS-Covalent Linker
300 Å 2% NH2-SAMMS-Covalent Linker

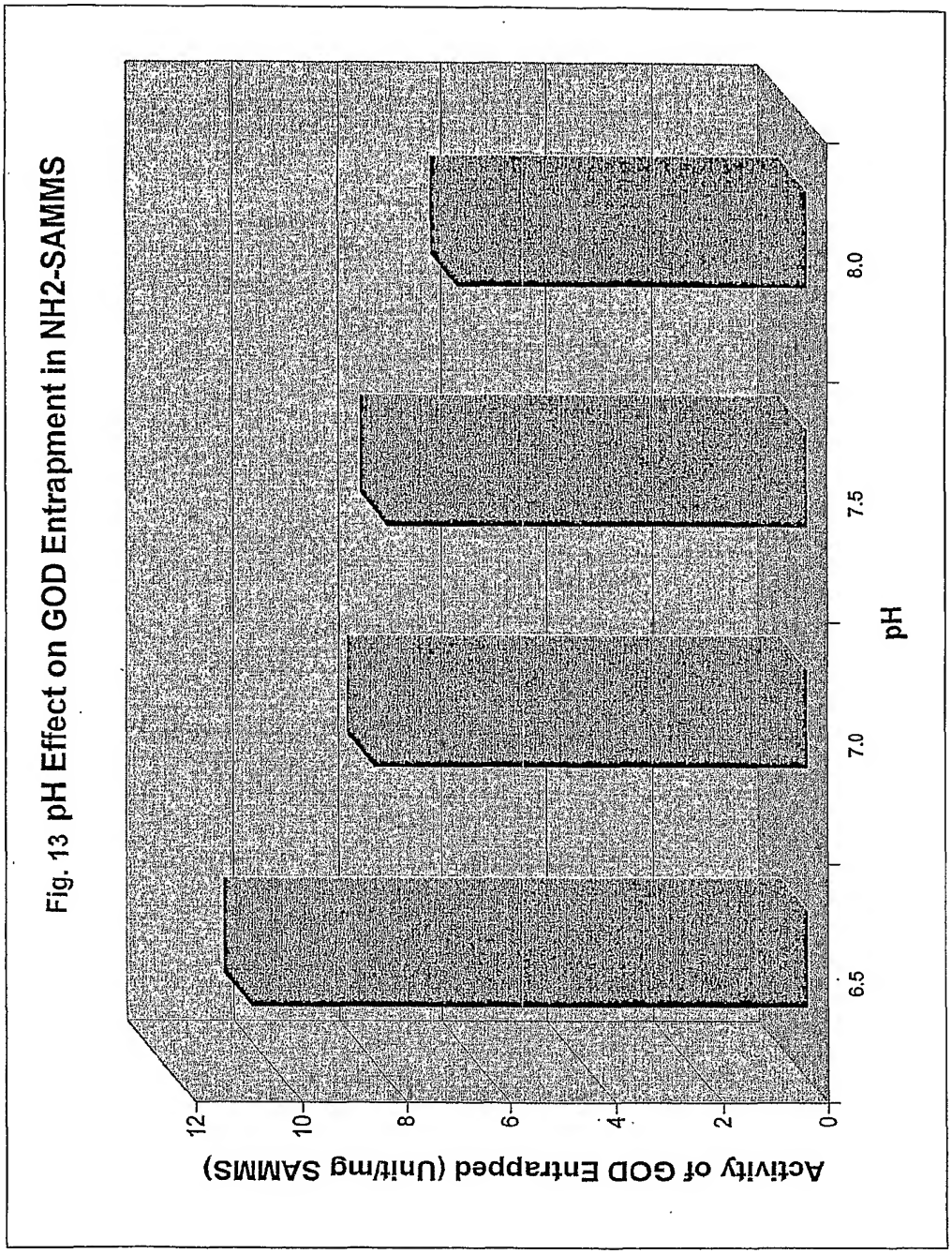

Fig. 13 pH Effect on GOD Entrapment in NH2-SAMMS

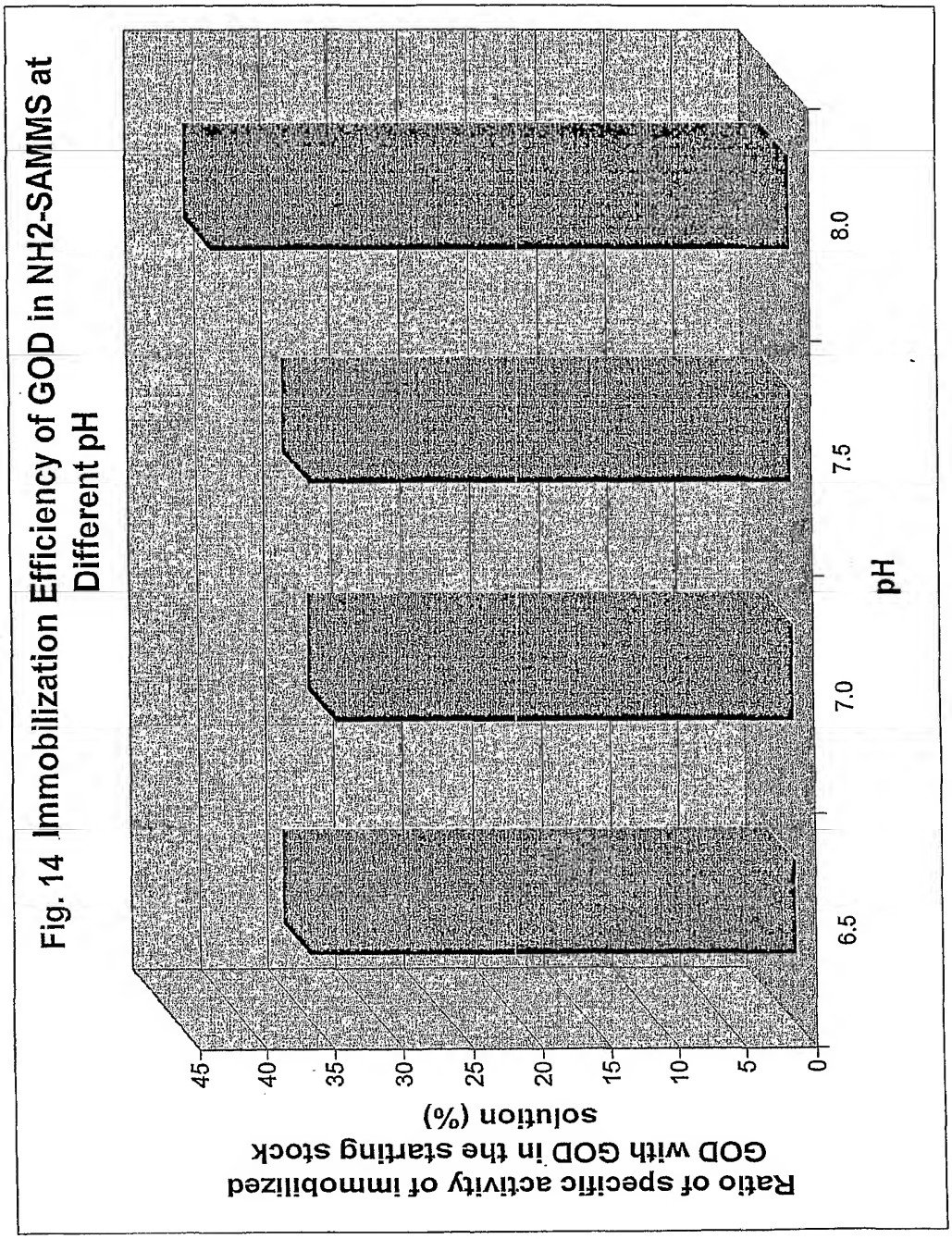

Fig. 14 Immobilization Efficiency of GOD in NH2-SAMMS at Different pH

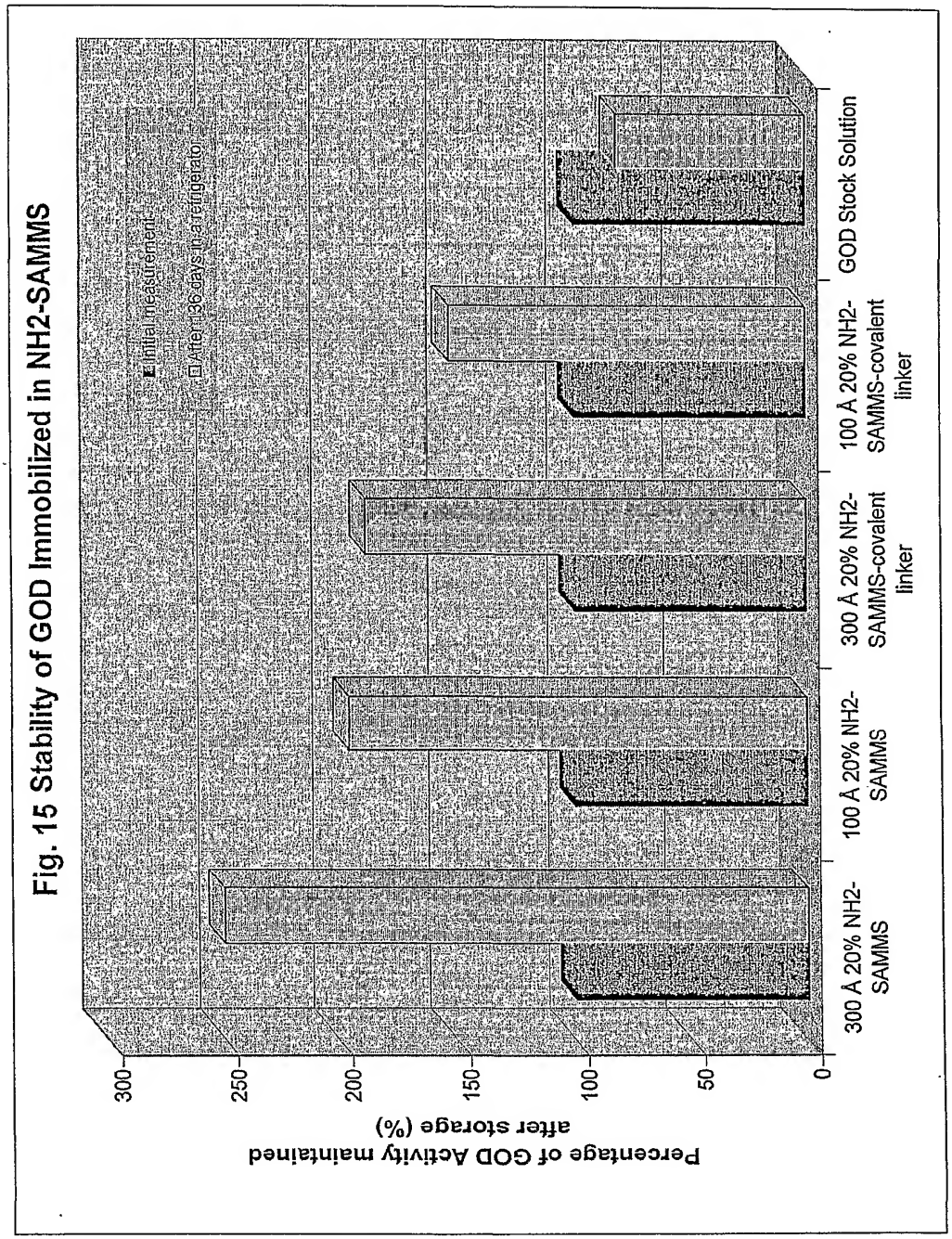
Fig. 15 Stability of GOD Immobilized in NH2-SAMMS
Initial measurement
After 136 days in a refrigerator
Percentage of GOD Activity maintained after storage (%)
300
250
200
150
100
50
0
300 Å 20% NH2-SAMMS
100 Å 20% NH2-SAMMS
300 Å 20% NH2-SAMMS-covalent linker
100 Å 20% NH2-SAMMS-covalent linker
GOD Stock Solution

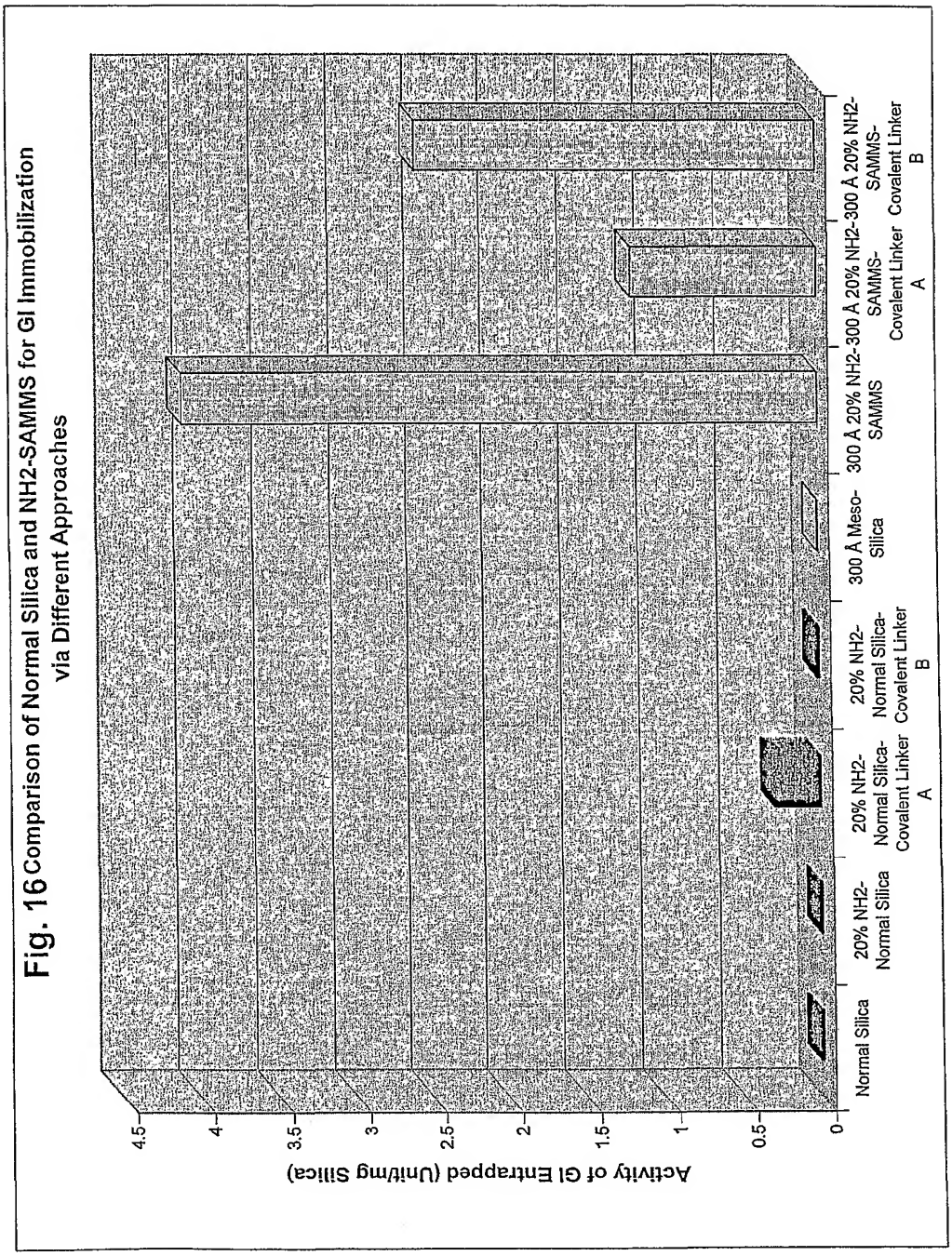

Fig. 16 Comparison of Normal Silica and NH2-SAMMS for GI Immobilization via Different Approaches

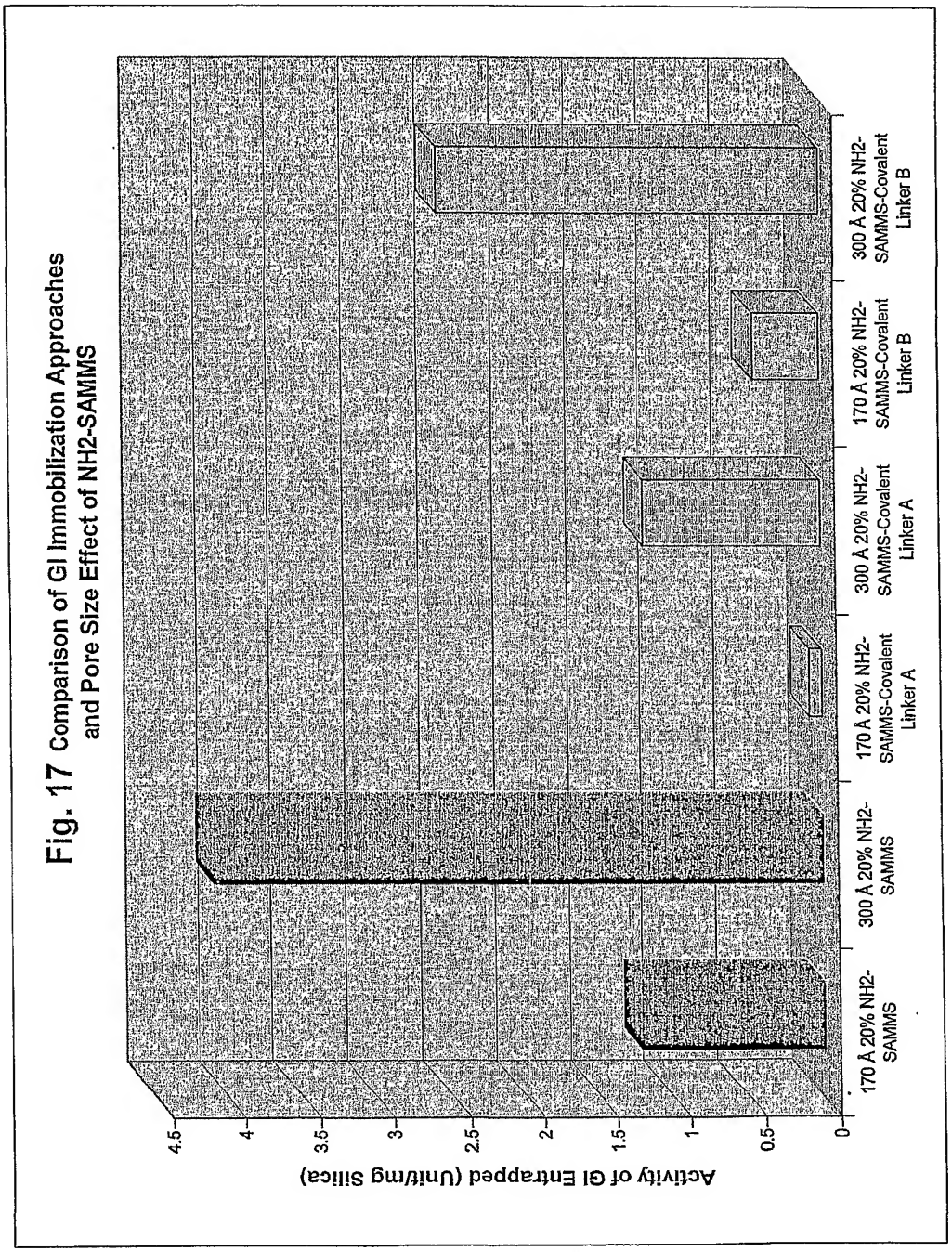

Fig. 17 Comparison of GI Immobilization Approaches and Pore Size Effect of NH2-SAMMS

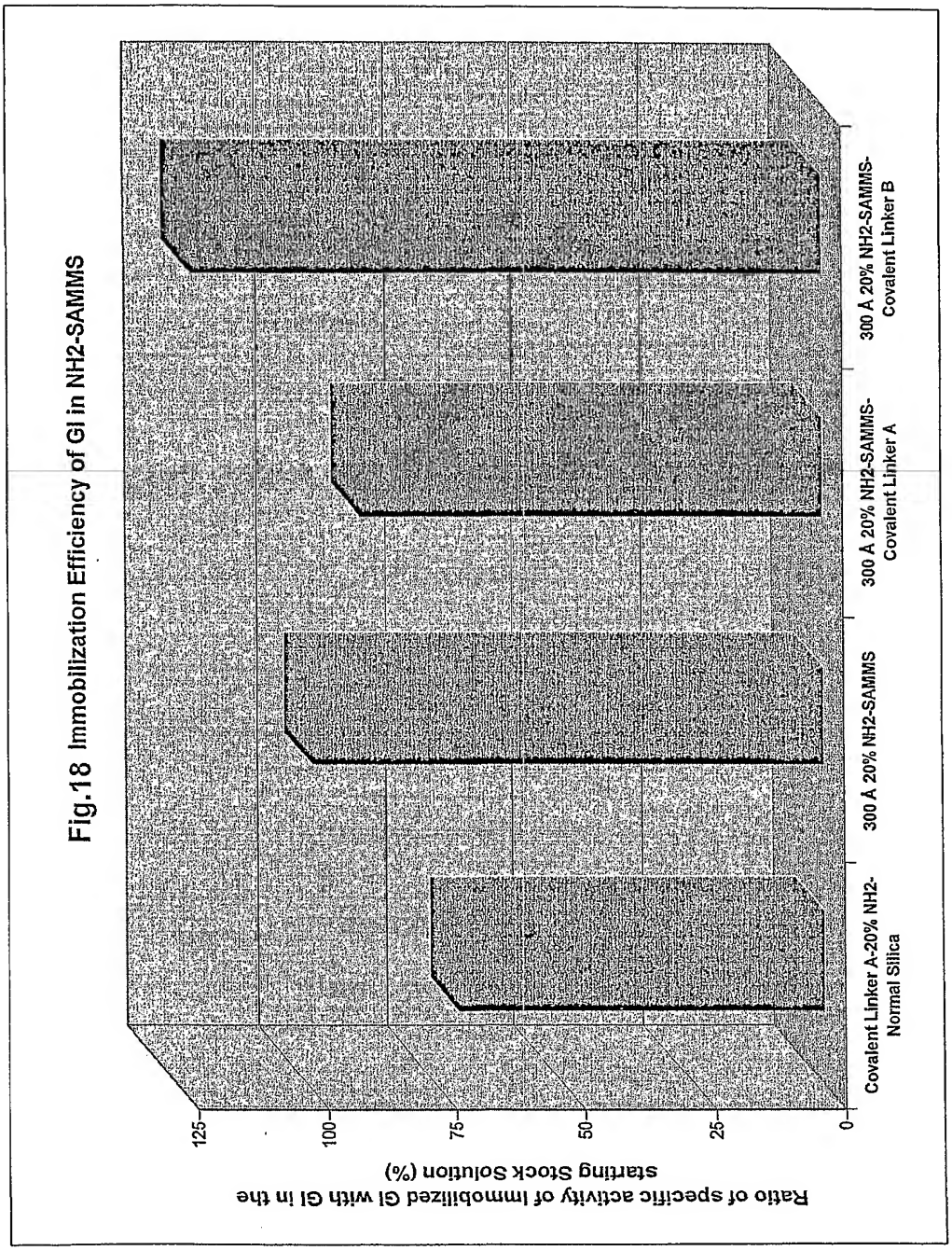
Fig.18 Immobilization Efficiency of GI in NH2-SAMMS
Ratio of specific activity of immobilized GI with GI in the starting Stock Solution (%)
125
100
75
50
25
0
Covalent Linker A-20% NH2-Normal Silica
300 A 20% NH2-SAMMS
300 A 20% NH2-SAMMS-Covalent Linker A
300 A 20% NH2-SAMMS-Covalent Linker B